PLASMODIUM FALCIPARUM CIRCUMSPOROZOITE PROTEIN: TARGET OF CELLULAR IMMUNITY AGAINST TRAVERSED HEPATOCYTES AND NATURALLY INDUCED MEMORY RESPONSES

by

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A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland

April 2015

ABSTRACT

Vaccines against exoerythrocytic stages of malaria remain ineffective in the face of limited knowledge on processing and presentation of parasite antigens in the liver. Here, we show that vaccine candidate circumsporozoite protein (CSP) is deposited and retained in human hepatocytes traversed by *P. falciparum* sporozoites for an extended time. Therefore, two reservoirs of hepatocytes contain malarial antigens: invaded and traversed cells. We demonstrate that CSP in traversed cells is long-lived, resides within several dynamic cellular compartments, and its turnover is regulated by distinct proteolytic systems, including the proteasome, lysosome and autophagy. We provide the first demonstration of endogenous processing of a malaria liver stage antigen by non-infected, non-professional antigen-presenting cells (APCs) through multiple proteolytic systems. We propose that these processes are likely to generate a variety of antigenic products from CSP, as well as other sporozoite antigens shed into traversed cells, able to affect the effector phase of anti-malarial immunity.

Development of *Plasmodium* parasites in the liver can be controlled by cytotoxic T lymphocytes (CTLs) recognizing malarial antigenic peptides presented by the major histocompatibility complex (MHC) I on the surface of hepatocytes. It is unknown if breaching of the plasma membrane during sporozoite traversal affects the ability of traversed hepatocytes to stimulate CTL responses, and if traversal affects the immunogenicity of nearby "non-traversed" hepatocytes in the infected liver. We show that traversed cells remain viable and competent to present endogenously processed antigen to CD8+ T cells. Stimulated CTLs undergo the complete program of antigen-

specific activation, including cytokine secretion, cytotoxic granule release, and proliferation. Soluble factors released by either traversed hepatocytes or by T cells activated on traversed hepatocytes do not impose traversal-specific in-*trans* effects on CTL activation. Finally, traversed hepatocytes endogenously presented a C-terminal epitope of CSP to specific CTLs, stimulating IFNγ production. Our data propose a novel view of traversed cells as a potential site of parasite-specific immune activation.

Since protective immune responses achieved in experimental vaccination differ dramatically from the partial protection naturally acquired in malaria-endemic regions, we next investigated the repertoire of HLA-A*02-restricted CSP-specific CD8+ memory T cells in PBMCs from adults living in malaria-endemic Mali. While plasma of 28/45 of Malian adults included in the study had detectable CSP antibodies, PBMCs from only 10/45 had functional *ex vivo* recall responses to CSP. Compared to malaria-naïve US volunteers, PBMC samples from Malian adults exhibited altered composition of the T cell compartment and increased expression of T cell exhaustion marker, PD-1. Our preliminary data demonstrate that T cell activation induced via TCR triggering could be facilitated by PD-1-specific, neutralizing monoclonal antibodies. However, it still needs to be discovered if malaria-specific memory T cell responses can be enhanced upon PD-1 blockade in PBMCs from donors living in malaria endemic areas.

Together, we propose a role for sporozoite traversal in CD8+ T cell responses against liver stage infection, and that novel vaccination strategies are needed to overcome immune dysregulation in T cell compartment found in populations from malaria-endemic regions.

ACKNOWLEDGEMENTS

The completion of this work depended on many individuals at several institutions. I have relied on and thank people whom I have never met: study participants in Dangassa, Mali, and D. Mahamadou for collaborating on this project; G. Corradin, University of Lausanne for providing T cells. I thank current and former colleagues at the Johns Hopkins University who have provided reagents and technical assistance: J. Bream, A. Durbin, G. Ketner, V. Levitsky, F. Zavala, and H. Zhang. I thank members of my thesis advisory and defense committees: J. Bosch, I. Coppens, A. Cox, A. Durbin, M. Hardwick, L. Luznik, S. Sadegh-Nasseri, P. Sinnis, and F. Zavala. I thank current and former colleagues in the lab who have contributed directly to this work: Z. Arany, P. Dumoulin and J. Ma. I thank my thesis advisor, J. Levitskaya for research opportunities and mentoring, and rotation advisors G. Dimopoulos and G. Ketner. Finally, I thank the Johns Hopkins Malaria Research Institute (JHMRI) for funding; Sommer Scholars Leadership program for training and funding.

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Chapter 1. INTRODUCTION

1.1 Malaria is a public health challenge

Malaria remains a major global public health burden, with one half of the world's population living in areas with ongoing transmission. The parasitic infection caused an estimated 198 million cases and 584,000 deaths in 2013, 78% of which were children under the age of five in sub-Saharan Africa (World Health Organization 2014). These recent morbidity and mortality rates are the result of vast progress in the last decade due to improvements in interventions and access to them. However, significant challenges remain to reach the WHO vision of "A world free of malaria." Future malaria programs are currently at a crossroads as in 2015, the WHO will establish a new strategy to reach 2030. The development of an effective vaccine against malaria will be a high priority, cost-effective strategy in reaching these goals. Efforts to generate a protective vaccine are hindered by many factors, including but not limited to antigenic variation, complex transmission patterns, and an inadequate understanding of immune mechanisms and correlates of protection, especially against pre-erythrocytic infection.

1.2 *Plasmodium* species

The causative agent of malaria is an apicomplexan parasite of the genus *Plasmodium*. Human malaria results from infection with one of five species through transmission by female *Anopheles* mosquitoes: *P. falciparum*, the cause of most severe disease, *P. vivax,* notable for the persistence of long-term, drug-resistant hypnozoite

reservoirs in the liver, *P. malariae, P. ovale,* and more recently recognized, the zoonotic *P. knowlesi* (Kantele & Jokiranta 2011).

1.3 *Plasmodium* life cycle

Plasmodium parasites have a complex life cycle alternating between sexual development in the mosquito and asexual development in the vertebrate host. When an infected female *Anopheles* mosquito harboring mature sporozoites in the salivary gland takes a blood meal to support egg production, sporozoites are deposited into the skin. These highly motile sporozoites (reviewed in (Montagna et al. 2012)) enter the bloodstream to reach their target organ, the liver, within minutes to hours (Yamauchi et al. 2007). It is believed that sporozoites continue to migrate until ultimately invading a single hepatocyte, within which they will develop and replicate inside a parasitophorous vacuole (PV) over seven days in the case of *P. falciparum* (reviewed in (Mikolajczak & Kappe 2006)). Thousands of mature merozoites are released from the hepatocyte to invade erythrocytes, establishing the symptomatic blood stage infection and exponentially amplifying parasite load, as each infected erythrocyte yields 16-32 daughter merozoites (reviewed in (Hafalla et al. 2011)). With each asexual cycle, the host develops characteristic periodic fevers, but symptoms may vary from asymptomatic to mild to severe as parasite load increases. In response to poorly understood stimuli, a portion of asexual parasites differentiate into male or female gametocytes, transmissible to the mosquito (reviewed in Drakeley et al. 2006). Upon taking a blood meal, female mosquitoes ingest gametocytes and foster the fertilization and development into ookinetes in the midgut. Ookinetes breach the midgut epithelium, transform into oocysts and then into sporozoites that travel to the salivary gland for transmission to the next host,

over two to three weeks. Utilizing a complex life cycle involving two hosts, three developmental sites (mammalian liver and blood, mosquito midgut), and several weeks' time, it is an evolutionary marvel that malaria could present such a grave public health concern.

1.4 Malaria pathogenesis

Symptoms of *P. falciparum* infection are associated with erythrocytic infection and characteristically include cyclical fevers, commonly accompanied by chills, sweats, headache, and malaise, attacking with every 48-hour replication cycle. More severe malaria may be associated with anemia, coma, respiratory and neurological complications that can ultimately lead to death. These symptoms can represent the result of a complex balance between parasite replication and the immune response (reviewed in (Hisaeda et al. 2005)). Pathogenesis partially results from the cytoadherence of parasitized erythrocytes to the vascular endothelium, mediated by expression of "knobs" of *Pf*EMP1 on the erythrocyte membrane. This cytoadherence is associated with cerebral and placental malaria (reviewed in Maier et al. 2009). Most uncomplicated infections are resolved following treatment with artemisinin-based combination therapies (reviewed in (Visser et al. 2014). However, emergence of drug-resistant strains is an increasing concern, emphasizing the importance of developing a preventive vaccine.

1.5 Sporozoite and liver stage biology

While the erythrocytic forms of *Plasmodium* were discovered in 1880, the clinically silent, pre-erythrocytic stage was not located in the liver until almost 70 years later. Once in the liver, productive pre-erythrocytic development requires sporozoite

invasion of a single hepatocyte with formation of a PV, consisting of host- and parasite-derived membranes, (reviewed in Lindner et al. 2011). Schizogony, replication, cytokinesis, and differentiation into thousands of merozoites follows as the PV grows to larger than the host hepatocyte (Sturm et al. 2009). Thousands of merozoites are then released from the hepatocyte to invade erythrocytes (reviewed in Mikolajczak & Kappe 2006).

Prior to exoerythrocytic form (EEF) development, however, the sporozoite must actively overcome physical barriers and innate immune responses in order to reach the target liver parenchyma (Hopp & Sinnis 2015, reviewed in Sinnis & Zavala 2012). Studies of early interactions between the sporozoite and mammalian host have been relatively limited until recently with the advancement of intravital imaging techniques (Tavares, Formaglio, Medvinsky, et al. 2013), but have begun to elucidate the events crucial for infection and priming of the adaptive immune response that occur even prior to sporozoites reaching the liver.

1.6 Sporozoite motility

1.6.1 Motility in apicomplexa

Plasmodium belongs to the phylum apicomplexa, alongside other human and livestock pathogens *Cryptosporidium, Toxoplasma gondii, Eimeria* and piroplasms (reviewed in Montagna et al. 2012). All are unicellular, obligate intracellular parasites with motile invasive forms that must penetrate target organs for replicative infection. They share similar structural features that enable such motility: a polarized, elongated morphology and secretory apical organelles micronemes and rhoptries containing homologous proteins, and lack of cilia or flagella (Carruthers & Tomley 2008; Montagna

et al. 2012). This biology is essential for host cell recognition and attachment, establishment of the parasitophorous vacuole, and for remodeling of the host cell post-invasion.

1.6.2 Sporozoite journey to the liver requires active motility

Because sporozoites were found in human blood immediately during and after blood feeding, it was initially thought that sporozoites were injected directly into the bloodstream (Fairley 1947). Later, Vanderberg suggested that sporozoites must be actively motile during parts of their journey to complete their "adventurous existence," between the mosquito and mammalian host (Vanderberg 1974). He realized that while some sporozoites were deposited directly to the bloodstream, and those could be passively carried to the liver with blood flow, most sporozoites were probably injected into the skin, from which they must actively escape. Several studies in rodents have since demonstrated that most sporozoites are indeed injected into the dermis, and must actively migrate to the bloodstream (Sidjanski & Vanderberg 1997; Matsuoka et al. 2002; Amino et al. 2008). While about one hundred sporozoites are injected during a mosquito bloodmeal (Medica & Sinnis 2005), far fewer actually reach the target organ, the liver. About 20% of sporozoites remain in the proximal draining lymph node (Amino et al. 2006; Yamauchi et al. 2007), and up to 5% stay the skin, where some may partially develop into EEFs (Gueirard et al. 2010; Voza et al. 2012).

On a cellular level, *Plasmodium* sporozoites use two types of motility to travel from the dermal injection site to their ultimate site of replicative infection, hepatocytes: gliding and traversal (reviewed in (Sinnis & Zavala 2012)). Both methods depend on

secretion of micronemal components and have been observed experimentally *in vitro* and *in vivo*.

Gliding, substrate-dependent motility without cilia or flagella, or major changes in cell morphology, is controlled by a subpellicular actinomyosin motor linked to the sporozoite surface by thrombospondin-related anonymous protein (TRAP) (reviewed in King 1988; Kappe et al. 2004). Gliding is observed experimentally as circular movement on glass, thought to reflect a similar pattern of gliding over a cell surface. Therefore, gliding is frequently used as an *in vitro* measure of sporozoite viability and motility (Lupton et al. 2015). TRAP is required for both gliding motility and productive infection. It is shed from the sporozoite surface, and is thus under investigation as a vaccine candidate (Sultan et al. 1997; Ejigiri et al. 2012).

Traversal is the transmigration of sporozoites in and out of cells, and has been observed in many cell types (Vanderberg et al. 1990; Mota et al. 2001; Coppi et al. 2007; Amino et al. 2008) as well as in *Toxoplasma* and *Eimeria* sporozoites (Speer et al. 1997; Danforth et al. 1992). *In vivo*, *Plasmodium* sporozoite traversal is critical for dermal exit (reviewed in Sinnis & Zavala 2012). While traversal occurs extensively *in vitro* through monolayers of a single cell type, its function is unclear. Both gliding and traversal can be inhibited *in vitro* by anti-sporozoite antibodies in a dose dependent manner (Behet et al. 2014).

In addition to gliding and traversal, A third distinct motile behavior is used for cell invasion, which results in formation of the PV, the ultimate destination of the sporozoite.

1.6.3 *Plasmodium* traversal

The first observations of active cell traversal by *Plasmodium* sporozoites *in vitro* were performed with *P. berghei* in peritoneal macrophages (Vanderberg et al. 1990). Phase contrast videomicroscopy revealed several types of interactions between sporozoites and rodent macrophages, which were described as: no interaction, interaction without entry, killing by the macrophage extracellularly, phagocytosis, phagocytosis followed by escape, active penetration, and death of the macrophage following entry and exit. Interestingly, these characterizations included both active and passive interactions whereas previously, it had been thought that sporozoites were passively phagocytosed by Kupffer cells This and other studies suggesting that sporozoites enter the liver parenchyma via Kupffer cells (Barnwell 2001; Frevert et al. 2006) raise questions of how sporozoites survive within these macrophages, cells specialized to phagocytose and destroy pathogens. It also presents the possibility for antigen presentation of sporozoite-secreted proteins by macrophages.

Cell traversal was subsequently observed in hepatocytes prior to invasion, which has been proposed to serve multiple roles: "activating" sporozoites for invasion, enhancing susceptibility of hepatocytes to subsequent invasion by releasing hepatocyte growth factor (HGF), immune evasion, by de-activating innate immune responses of Kupffer cells, and depositing antigen in non-target cells as a decoy (Mota et al. 2001; Mota et al. 2002; Mota & Rodriguez 2004; Zheng et al. 2014). Understanding of cell traversal by sporozoites was enhanced by the identification and disruption of sporozoite microneme protein essential for traversal (SPECT, SPECT-2) (Ishino et al. 2004). Sporozoites deficient in SPECT and SPECT-2 proteins are competent to invade

hepatocytes at the same rate as wild-type sporozoites *in vitro*, questioning the hypothesized requirement for traversal prior to invasion (Mota et al. 2002).

Recent live imaging studies in mice showed the first *in vivo* evidence of sporozoite traversal through hepatocytes (Tavares, Formaglio, Thiberge, et al. 2013). To reach productively invade, sporozoites breached the sinusoids through different mechanisms involving traversal: 53% traversed through endothelial cells lining the sinusoids, 24% through Kupffer cells, and the remaining minority crossed intercellular gaps independently of traversal. These *in vivo* studies also challenged the sporozoite "activation" hypothesis (Mota et al. 2002).

There has been extensive speculation about the role of traversal in the life cycle of the parasite. With new innovations in live imaging of small animals using spinning disk confocal microscopy, this body of knowledge will continue to grow quickly. While much research has focused on the utility of traversal for the sporozoites, we believe our studies are among the first to address the effect of traversal through hepatocytes on the host.

1.7 Circumsporozoite protein (CSP)

Closely linked to traversal activity is circumsporozoite protein (CSP). Long since referred to as CSP, the major sporozoite surface protein and immunodominant antibody target in mice was first identified in *P. berghei* as Pb44 (Yoshida et al. 1980; Aikawa et al. 1981). Anti-Pb44 monoclonal antibody 3D11 was observed to bind a protein coating the surface of mature, salivary gland sporozoites, as well as EEFs from *P. berghei* infected rats until 24 hours post-infection, but not later. These antibodies were shown to have a function in inhibiting the infectivity of sporozoites injected onto Balb/c mice. Therefore, CSP was quickly under investigation as a subunit vaccine, as whole sporozoite

vaccines were effective, but impractical (Yoshida et al. 1981). More than forty years later, CSP remains the single most advanced vaccine candidate.

1.7.1 CSP has several essential roles

Comprising up to twenty percent of total protein in mature sporozoites (Yoshida et al. 1981; Cochrane et al. 1982), CSP has several key functions for the sporozoite both in the mosquito and mammalian host (Ménard et al. 1997; Pancake et al. 1992; Stewart et al. 1987), making it essential for infectivity. It is first expressed at high levels in the mosquito midgut oocyst and is required for sporogony and sporozoite invasion into mosquito salivary glands (Nagasawa et al. 1987; Posthuma et al. 1988; Myung et al. 2004). Once injected into the skin, it is important for gliding motility (Stewart & Vanderberg 1988) and adhesion to the target hepatocyte through recognition of heparan sulfate proteoglycans (HSPGs)(Pancake et al. 1992; Cerami et al. 1992; Pinzon-Ortiz et al. 2001).It is further utilized in sporozoite invasion of hepatocytes (Stewart et al. 1986). *P. falciparum* CSP continues to be expressed for the first five days of EEF development (Sacci et al. 2006; Mikolajczak et al. 2011). Its expression on the sporozoite surface as well as inside infected hepatocytes make CSP a potential target of specific antibody responses during invasion, as well as MHC-class-I restricted recognition by cytotoxic T lymphocytes (CTLs) inside hepatocytes.

1.7.2 CSP functional domains

The general structure of CSP, consisting of a tandem repeat region flanked by N- and C-terminal domains, is well conserved among different *Plasmodium* species, suggesting essential roles for each domain. Elucidation of the conformation of CSP has

been challenging to crystallographers because of its unusual, repetitive structure (Doud et al. 2012).

The N-terminus contains a signal sequence and Region I (RI), consisting of 5 conserved amino acid residues, KLKQP, in all species studied except *P. gallinaceum* (NLNQP). The N-terminus has roles in cell adhesion, binding to mosquito salivary glands (Sidjanski et al. 1997; Myung et al. 2004) and to HSPGs in the liver (Rathore et al. 2002; Rathore et al. 2005). Cleavage occurs at RI by a parasite protease upon sporozoites contact with host hepatocytes (Coppi et al. 2005; Coppi et al. 2011). Functionally, this cleavage event switches sporozoite behavior from "adhesive," to "invasive," and is important for hepatocyte invasion (Coppi et al. 2011). Antibodies recognizing the RI site have been shown to inhibit cleavage of CSP and impair invasion *in vitro* infection and well as infection *in vivo* (Espinosa et al. 2015).Therefore, induction of N-terminal RI-specific antibodies may be a desirable target of vaccination.

Comprising 42% of the 3D7 reference strain of *P. falciparum,* the central tandem repeat region is a B-cell immunodominant stretch of 38-43 species-specific tetrameric repeats (Gandhi et al. 2014). The sequence bears no homology to other known proteins, though other *Plasmodium* proteins do contain tandem repeat sequences that are also immunogenic (Anders et al. 1986). While the exact function of the repeat region is unknown, it is essential in sporozoite development, as repeat deletion mutants in *P. berghei* fail to mature from oocysts to sporozoites (Ferguson et al. 2014). It is speculated to have a role in high avidity, multivalent interactions with glycosaminoglycans (GAGs) (Rathore et al. 2001).

Finally, the C-terminus of CSP contains a type I thrombospondin (TSR) domain that binds to HSPGs and HepG2 cells in conjunction with the N-terminus (Ying et al. 1997). While TSRs are common in other organisms and is found in TRAP, the CSP TSR is divergent (Tossavainen et al. 2006). The utmost 3' end of CSP contains a predicted canonical glycosylphosphatidylinositol (GPI) anchor.

1.7.3 CSP as target of adaptive immunity

CSP is highly immunogenic, inducing antibody responses in natural infection and in sporozoite-based vaccination, making it a primary vaccine target (Nardin et al. 1999; Birkett et al. 2002). The central repeat region contains an immunodominant B cell epitope (Zavala et al. 1983; Zavala et al. 1985a), to which anti-repeat antibodies inhibit hepatocyte invasion, justifying its inclusion in antibody-inducing vaccines (Nussenzweig & Nussenzweig 1989; Persson et al. 2002). The C-terminus contains important, polymorphic T-cell epitopes Th2R (aa 326-343) and Th3R (aa 361-380) (Good et al. 1988; De Groot et al. 1989; Zevering et al. 1994). MHC class I-restricted CSP-derived T cell epitopes and their significance to natural infection and vaccination will be discussed further below.

1.7.4 Genetic diversity in CSP

In the design of CSP-based vaccines, it is essential to consider the genetic variation often detected across *Plasmodium* strains. Overall evidence of the role of genetic polymorphisms in clinical outcomes of infection and protection following immunization vary across studies, however. In an in-depth prospective study of children in Mali, no associations were found between sequence variations in Th2R and Th3R and clinical malaria episodes, suggesting a lack of allele-specific protective immunity against

CSP in this region (Gandhi et al. 2014). While sequence variation is found in these regions, CTLs specific to this region can be cross-reactive for other alleles (Lyke et al. 2005). Follow-up studies to RTS,S trials in different settings have revealed different results, suggesting that strain-specific sequence variation in CSP was or was not implicated in vaccine failure (Waitumbi et al. 2009; Macete et al. 2007; Alloueche et al. 2003).

There are several possible explanations for the widespread diversity in CSP. Sequence variation may result from a combination of geographic separation of isolates (Tanabe et al. 2013) and selective pressure (Hughes 1991; Escalante et al. 2002). The variable number of repeats could be an immune evasion mechanism by the parasite by enhancing a non-protective response (Schofield 1990) or dictate the stability of CSP (Escalante et al. 2002). SNPs could result in generation of sequences giving rise to altered peptide ligands (Plebanski, Lee, et al. 1997; Gilbert et al. 1998), or reflect adaptation to bind salivary glands of different mosquito species (Kumkhaek et al. 2005). At present, the importance of genetic diversity in CSP in protective immunity is largely unclear.

1.7.5 CSP is shed by motile sporozoites

CSP is actively shed by gliding sporozoites *in vitro* (Stewart & Vanderberg 1988; Stewart & Vanderberg 1992) and is speculated to be retained in traversed cells. However, this has not been formally demonstrated. Prior to defining traversal as a cell migratory activity, CSP was observed microscopically in the cytosol of invaded cells (Hügel et al. 1996). Hügel *et. al.* concluded that this CSP was a result of protein exported from inside the PV. However, with more recent understanding of traversal, another possibility is that CSP was shed during sporozoite traversal prior to, or after the hepatocyte had been

invaded by another (or the same) sporozoite. TRAP and erythrocyte-binding antigen 175 (EBA-175) have also been found in trails of shed protein and lipids from motile sporozoites and are potentially deposited in traversed hepatocytes as well (Mota et al. 2002). Thus, we speculated that non-infected traversed cells might serve as a reservoir of deposited malarial antigen in close proximity to the site of replicative infection in the liver. This would present two distinct possibilities for the immune response: serving a benefit to the host, by enhancing T cell activation, or a detriment, diverting specific T cells away from EEFs.

1.7.6 CSP in immune evasion

CSP is expressed at high levels, conserved among species and strains, and is a protective antigen in experimental animal vaccination. High levels of anti-CSP IgG have long been detected in naturally exposed individuals in hyperendemic regions (Nardin et al. 1979; Offeddu et al. 2012). Yet, these antibodies do not afford protection in endemic settings (Hoffman et al. 1987), possibly because antibody titers are not induced or maintained at high enough levels to neutralize sporozoites. Together, these paradoxical features suggest a possible function of CSP in immune evasion (Schofield 1990), and could in part explain the poor immunity that results from natural infection.

Besides the strong but ineffective immune responses against CSP, there are several lines of experimental evidence supporting a role for CSP in immune evasion. In infection of human hepatoma cell line HepG2 with *P. berghei,* CSP was implicated in controlling protein synthesis in non-invaded cells (Frevert et al. 1998). In this study, completed prior to the identification of cell traversal in 2001, CSP was shown to arrive in the cytoplasm of non-invaded cells by translocation through the plasma membrane by

"extracellular attached sporozoites." It is now more commonly accepted that sporozoites shed CSP into the cytosol while traversing through a cell. These CSP-positive cells did not incorporate tritiated leucine from the culture medium immediately after co-cultivation with sporozoites, interpreted as CSP-directed shutdown of protein synthesis.

CSP has been reported to specifically interfere with several functions of the host hepatocyte important in inflammation, by entering the cytoplasm following export from the PV in invaded hepatocytes and competing with NFκB for nuclear import (Singh et al. 2007). However, we recently addressed several of these possible CSP-driven perturbations relevant to antigen presentation in the relevant cell type, human hepatocytes, using HC-04 cells. Our data show that HC-04 hepatocytes expressing either full-length or cleaved CSP maintain unaltered surface expression of MHC class I and transcript levels of other components of the MHC class I processing pathway (Ma et al. 2013). Altogether, while there is evidence for a role of CSP in alteration of host cell function and immune evasion, some studies warrant re-interpretation following newer findings about cell traversal.

1.8 Immunology of natural malaria infection

Natural exposure to malaria results in the development of clinical immunity, incomplete protection that may prevent the development of malaria symptoms by limiting, but not completely preventing infection (Offeddu et al. 2012). In highly endemic regions, exposed individuals develop partial clinical immunity with age and continued exposure, resulting in decreased illness and mortality while experiencing repeated re-infection (Baird et al. 1998). The acquisition of immunity appears to depend on the overall accumulation of exposure: in regions of high transmission, young children bear the

biggest disease burden, which wanes with age, while in lowly endemic areas, individuals of all ages are affected (Miller et al. 1994). Adults in endemic regions are often asymptomatic yet parasitemic, providing ongoing parasite reservoirs for continued transmission to the community. As of yet, no correlates or signatures of protective immunity have been defined in natural exposure. While more robust immune responses are directed against blood than liver stage antigens, which have a higher level and longer duration of exposure, our studies focus on the less understood responses to liver stage antigens.

1.8.1 Natural infection: Humoral responses

Antibodies to liver stage antigens, particularly CSP, are frequently detected in children and adults in malaria-endemic regions (reviewed in Offeddu et al. 2012). Serum samples from exposed individuals bind to the sporozoite surface, reflecting the pattern of staining observed using monoclonal antibodies against CSP. The percentage of populations in endemic regions whose sera recognize sporozoites increases with age but independently of current blood stage infection. Most studies of antibody responses against CSP have focused on IgG specific to peptides representing the central repeat region, detection of which generally servers as a surrogate for the whole protein. However, at least one study, of a population in endemic Gabon, detected antibodies against CSP RI in a small proportion of donors whose serum lacked reactivity with the central repeat (Del Giudice et al. 1988). A protective function of antibodies against the N-terminus were suggested by a report of association between antibodies to this region with protection from clinical malaria in children in Tanzania (Bongfen et al. 2009). While a causal relationship has yet to be established, it would be reasonable to expect that

antibodies recognizing the N-terminus could have different functions than those to the repeat region because of the distinct functions of each domain of the protein. Thus, it may be important for studies of humoral immunity to CSP to consider responses to each CSP domain, namely N- and C-termini as well as the repeat region. As such, our studies have focused on finer specificities of antibodies to CSP. As of yet, however, antibodies to CSP appear to signify exposure to malaria rather than protection.

Other sero-epidemiological studies have investigated naturally induced antibody responses to vaccine candidates including LSA-1, LSA-3 TRAP, EXP-1, and STARP individually, and some have utilized high throughput methods of screening hundreds of *Plasmodium* antigens (reviewed in Doolan 2011). A recent study of Malian children associated protection with the breadth of antigens recognized instead of responses to single antigens (Daou et al. 2015), similar to what has been observed in vaccination studies (Trieu et al. 2011).

1.8.2 Natural infection: Cellular responses

Because MHC class I-restricted CD8+ T cell responses are important in protection following vaccination (discussed below), the identification of protective class I-restricted T cell epitopes from liver stage antigens is a high priority for vaccine development. *Ex vivo* studies of naturally exposed individuals are technically difficult, as the frequency of specific T cells of interest are likely rare; a methodological study showed that at least three simultaneous approaches of re-activation of memory T cell responses *ex vivo* were required to sensitively identify *Plasmodium*-specific T cells in PBMCS from exposed individuals in The Gambia (Flanagan et al. 2001). Variety of HLA haplotypes and its high variability between different regions in endemic areas poses

another challenge, to predict which peptides epitopes are likely to be presented. Nonetheless, many studies have investigated HLA-restricted, liver stage specific T cell epitopes (Doolan et al. 1991; Sedegah et al. 1992; Aidoo et al. 1995; Blum-Tirouvanziam et al. 1995), to be discussed further in Chapter 4.

1.8.3 Natural infection: Immune dysregulation

Populations at risk of malaria often live in regions afflicted by poverty, malnutrition, and co-infection with chronic helminth infections or HIV. Therefore, natural immune responses to malaria occur in the context of other co-morbidities that can profoundly impair the immune response. Malaria itself manifests more as a chronic than acute infection in settings of repeated and recrudescent illness. There is a well-documented overall impairment of B-cell memory responses in natural exposure (Weiss et al. 2010; Liu et al. 2012) and exhaustion of T-cell responses, mediated by immune inhibitory receptors such as PD-1 (Horne-Debets et al. 2013). Overcoming these challenges to mounting effective immune responses will be critical in designing effective vaccines for malaria in the developing world.

1.9 Malaria liver stage vaccines

Vaccines targeting the pre-erythrocytic stage of malaria infection are an optimal approach because abrogation of this pre-symptomatic infection blocks the onset of clinical symptoms of malaria as well as transmission. However, complete protection against liver stage infection is challenged by the fact that only one sporozoite that invades a hepatocyte can be sufficient to propagate infection. Therefore, a pre-erythrocytic vaccine must completely eliminate every single sporozoite or infected hepatocyte.

Research in small animals, non-human primates, and humans support the feasibility of a protective vaccine.

1.9.1 Attenuated sporozoite vaccines

The vaccine "gold standard," capable of generating sterile protection in humans is the irradiated sporozoite vaccine (IrrSpz) (Nardin et al. 1999). Protection following immunization of A/J mice with irradiated *P. berghei* sporozoites was first demonstrated in 1967 (Nussenzweig et al. 1967), followed shortly thereafter by successful immunization of Balb/c mice (*P. berghei, P. yoelii)* and rhesus macaques (*P. knowlesi*) (Gwadz et al. 1979). Radiation-attenuated *P. berghei* and *P. falciparum* sporozoites exhibit comparable motility, hepatocyte invasion rates, and early EEF development to wild-type sporozoites (Mac-Daniel et al. 2014; Silvie et al. 2002). The same vaccination approach has elicited protection in humans. The dosing requirements to show protection in humans is very high: protection may require at least 1,000 bites by infected, irradiated mosquitoes (*P. falciparum*, *P. vivax*), which clearly does not reflect the manner of natural infection by mosquito bite, and is not a practical vaccination strategy (Clyde, McCarthy, et al. 1973; Clyde, Most, et al. 1973; Clyde et al. 1975; Edelman et al. 1993).

Genetic attenuation is also under investigation following initial studies in mice inducing long-term immunity, though some have experienced problems with breakthrough infections due to insufficient attenuation (Mueller et al. 2005; Tarun et al. 2007; van Dijk et al. 2005). The first proof-of-principle infection of humans with p52-/p36- genetically attenuated *P. falciparum* sporozoites effectively induced anti-CSP antibodies that inhibited sporozoite invasion, as well as IL-2, TNFα, and IFNγ secreting CD4+ and CD8+ T cells upon re-stimulation with sporozoites or individual liver stage

antigens (Spring et al. 2013; Finney et al. 2014). However, one of six volunteers infected with the attenuated parasite developed blood stage parasitemia. Thus, future genetically attenuated parasites must be further attenuated to avoid causing illness.

A third approach to whole sporozoite vaccination is wild-type sporozoite infection under chemoprophylaxis (CPS), which requires 20-fold fewer mosquito bites for sterile protection than IrrSpz immunization (Nahrendorf et al. 2014). As chloroquine specifically kills erythrocytic stage parasites, this immunization strategy presumably exposes the vaccinee to the full breadth of liver stage antigens, as well as limiting the quantity of blood stage antigens.

Irradiation- and genetically-attenuated parasites proceed through early stages of liver stage development, expressing multiple sporozoite and liver stage antigens (SLA), but arrest prior to merosome maturation. CPS is the only whole sporozoite method of vaccination that proceeds to early blood stages, reviewed in (Nganou-Makamdop & Sauerwein 2013; Epstein & Richie 2013). Importantly, protection is not induced by inoculation with heat-killed sporozoites (Alger & Harant 1976), indicating that sporozoites must be viable for successful vaccination. Altogether, attenuated whole sporozoite vaccines clearly demonstrate that SLA can confer protection, but this method of vaccination is impractical. Thus, these findings have led to further investigation of subunit vaccination with single or multiple SLA.

1.9.2 Subunit vaccines

The most advanced subunit vaccine to date is the CSP-based RTS,S, which contains the central repeat and C-terminus of CSP fused to HBSAg. As protection has been achieved in experimental models in CSP-tolerized mice (Kumar et al. 2006), other

pre-erythrocytic antigens under study include thrombospondin-related adhesion protein (TRAP), liver-stage antigen (LSA), exported protein 1 (Exp-1), and cell-traversal protein for ookinetes and sporozoites (CelTOS) (reviewed in (Duffy et al. 2012)).

RTS,S/AS01

The RTS,S/AS01 vaccine, (named for Repeat, T-cell epitopes, fused to Hepatitis B S antigen, expressed in yeast with non-fused S antigen) developed in part by GlaxoSmithKline, has been tested in Phase III trials in Africa. The vaccine-like particle (VLP) displays the repeat and C-terminal domains of CSP fused to hepatitis B surface antigen (HBSAg) (Cohen et al. 2010). Clinical trials in Africa resulted in 50% protection in non-exposed volunteers (Kester et al. 2009), but only 30-45% in children, with fewer infants protected (6-12 weeks old) than older children (5-17 months). (Bejon et al. 2013; Olotu et al. 2013). A recent high throughput study of the breadth and magnitude of antibody responses to a large panel of liver- and blood-stage antigens following RTS,S vaccination showed decreased overall parasite-specific antibody responses in RTS,S vaccinated, versus control-vaccinated children in Mozambique (Campo et al. 2014). CSP was, expectedly, an exception. These findings suggest that the immune responses induced by RTS,S inhibit sporozoite invasion, thus blocking infection and exposure to other liver and blood stage antigens that would boost antibody responses. While the partial protection appeared to be associated with antibodies (Kester et al. 2009) and CSP-specific CD4+ T cells (Good & Doolan 2010) but not CD8+ T cells, correlates of protection have not been firmly defined.

Other pre-erythrocytic vaccines in clinical trials include PfCelTOS FMP012, CSVAC, and Ad35 vectored CS and RTS,S-AS-1 combined in heterologous prime-boost

schedule (Phase 1), and the ChAd63/MVA in Phase 2 (reviewed in (Arama & Troye-Blomberg 2014)).

1.10 Mechanisms of protective immunity following experimental liver-stage immunization

Depending on the experimental model, many immune components are implicated in the response to liver stage infection, including antibodies, CD4+ and CD8+ T cells, gamma delta T cells, NK, and NKT cells, (Duffy et al. 2012; Stanisic et al. 2013). Immune mechanisms required for protection have been shown to include antibodies, CD4+ and CD8+ T cells, interferon gamma (IFNγ) and/or tumor-necrosis factor alpha (TNFα) in different experimental mouse and non-human primate models (Doolan & Hoffman 2000). These studies illustrate the complex nature of the interactions involved in each host-parasite combination.

1.10.1 Vaccine-induced anti-sporozoite antibodies

There are several lines of experimental evidence supporting protective roles of antibodies in animal models. In early studies of irradiated sporozoite-based vaccination, sera of immunized animals and humans exhibited "sporozoite neutralizing activity" (SNA) that could inhibit sporozoite infectivity (Nussenzweig et al. 1969). Moreover, antibodies targeting CSP were specifically implicated in this neutralization. Passive transfer of monoclonal antibodies have provided protection against infection with *P. berghei* (Potocnjak et al. 1980) and *P. yoelii* (Charoenvit et al. 1991) sporozoites in mice and *P. falciparum*, *P. vivax,* and *P. knowlesi* in chimpanzees and monkeys (Nardin et al. 1982; Cochrane et al. 1982). Recently, high titers of anti-*P.falciparum* CSP antibodies

induced by vectored-immune prophylaxis have partially protected mice from infection with *P. berghei* chimeric parasites expressing *P. falciparum* CSP (Deal et al. 2014).

Possible functions of sporozoite-specific antibodies in protection against infection could include sporozoite killing through opsonization, inhibition of hepatocyte invasion, recognition and elimination of infected hepatocytes through antibody-dependent cellular cytotoxicity (ADCC), or recognition of mature merozoites and inhibition of erythrocyte invasion (reviewed in Casares & Brumeanu 2010). Antibodies against sporozoite surface proteins, including CSP, have been shown to inhibit sporozoite motility in the skin of mice and prevent invasion (Vanderberg & Frevert 2004; Vanderberg et al. 2007; Kebaier et al. 2009). The same has yet to be demonstrated in humans. However, *in vitro* studies have shown that human monoclonal antibodies against CSP generated from protected, vaccinated individuals inhibited sporozoite gliding on glass and invasion through HC-04 cells, in a dose-dependent manner (Clement et al. 2012). *In vivo,* passive transfer of these human anti-CSP antibodies protected humanized uPA-SCID mice from *P. falciparum* infection one day post administration (Foquet et al. 2013). The injection of 400 μg anti-CSP IgG led to plasma concentrations of about 500 - 1200 EU/mL in mice just prior to challenge, and was sufficient to protect mice from mosquito bite challenge.

Overall, evidence from mice and non-human primates showed that high levels of antibodies against CSP could mediate protection, and were thought to be sufficient following IrrSpz immunization in humans as well. But, even though RTS,S is associated with induction of anti-CSP antibodies, it provides only partial, short-term protection. Therefore, the sufficiency of CSP-specific antibodies in humans, whether induced following natural infection for immunization, remains unclear (Grüner et al. 2007; Behet

et al. 2014). As some RTS/S induced antibodies have sporozoite inhibitory activity *in vitro* and in humanized mice, it is possible that CSP antibodies following vaccination in humans are not generated at high enough levels, or titers decline too rapidly.

Several studies of immunized individuals associate protection with increased antibody responses to multiple sporozoite- and liver-stage antigens, instead of a single antigen (John et al. 2005; Trieu et al. 2011).

1.10.2 Vaccine-induced αβ T cells

Independently of antibodies, T cells have been implicated in protection following attenuated-sporozoite immunization. In early studies, the majority of *P. berghei* sporozoite-immunized B-cell deficient mice, 64%, were protected from sporozoite challenge, demonstrating a role for an antibody-independent mechanism of protection (Chen et al. 1977). In the same study, all T-cell deficient mice were susceptible to challenge post-vaccination with x-irradiated sporozoites. Selective depletion of CD8+ or CD4+ T cells has demonstrated important roles for each T cell subset, reviewed in (Duffy et al. 2012): CD8+ T cells are crucial in protection from *P. berghei* in rats (Schofield et al. 1987) and *P. yoelii yoelii* 17X NL in BALB/c mice (Weiss et al. 1988), while depletion of CD4+ subsets did not impair protection. A dependence on CD8+ T cells for protection has been demonstrated in several mouse studies (reviewed in (Hafalla et al. 2011)), albeit with different efficacy, suggesting that MHC class I haplotypes may play an important role in parasite-specific immune surveillance.

CD8+ T cells recognizing CSP in the context of MHC class I can afford against sporozoite challenge in mice. Mouse models have established requirements for very high amounts of circulating CSP-specific CD8+ T cells (exceeding 1% of total CD8+ cells)

(Schmidt et al. 2008), and the long-term presentation of CSP (Cockburn et al. 2010) to maintain these responses. High throughput studies using novel methods for identifying T cell specificities induced following natural exposure and vaccination in humans are under way (Grubaugh et al. 2013).

CD4+ T cells generally offer a supportive and secondary role in immunity to liver stage after CD8+ T cells, affording protection in the absence of CD8+ cells (Oliveira et al. 2008), and facilitating and maintaining CD8+ responses (Carvalho et al. 2002; Overstreet et al. 2011). Depletion of CD4+ T cells following immunization with *P. yoelii* sporozoites reduced protection, measured as liver parasite burden, though not to the degree of CD8+ depletion (Rodrigues et al. 1993). However, passive transfer of a CD4+ clone specific for a single non-CSP *P. berghei* sporozoite and blood stage antigen was sufficient to provide protection in mice (Tsuji et al. 1990).

In humans, IFNγ+ CD4+ and CD8+ T cells have been detected upon restimulation following sporozoite or CSP-based immunization (Moreno et al. 1991; Calvo-Calle et al. 2005; Seder et al. 2013; Spring et al. 2013; Roestenberg et al. 2009). But, their requirements for protection have not been demonstrated.

1.10.3 Vaccine-induced protective T cell effector functions

Multiple T cell effector mechanisms are implicated in liver stage protection, including direct cytotoxicity of infected hepatocytes, bystander killing through cytokine secretion, and helper T cell functions (reviewed in (Stanisic et al. 2013). The complexity apparent from these lines of evidence, mostly from rodent studies, indicates there are likely multiple, redundant mechanisms of protection (Doolan & Hoffman 2000).

Direct elimination of infected hepatocytes could occur through perforin-granzyme release or Fas/FasL interactions. The occurrence of such direct recognition and killing is supported by *in vitro* presentation of CSP from primary *P. berghei* infected hepatocytes to specific CD8+ T cells in a proteasome-dependent manner (Bongfen et al. 2007). Inhibition of *P. yoelii* development in bone marrow chimeric mice show a requirement for peptide recognition by cognate T cells on liver parenchyma supports this killing mechanism *in vivo* (Chakravarty et al. 2007). Furthermore, TAP-1 is required for activation of transferred CD8+ T cells specific for the H-2K^{b}-restricted SIINFEKL epitope within CSP of *P. berghei CS*^{5M} (Cockburn et al. 2011). Recent live imaging studies in mice co-infected with MHC-matched and mismatched *P. berghei* strains have demonstrated elimination of only hepatocytes infected with parasites contained cognate epitopes, demonstrating exclusively direct, and not bystander-mediated, killing of infected hepatocytes (Cockburn et al. 2014).

Multiple studies demonstrate important roles for pro-inflammatory cytokines in protection. Perforin-deficient and Fas-mutant C57BL/6 mice were both protected from *P. berghei* challenge following vaccination (Renggli et al. 1997). IFNγ depletion abrogated protection in *P. berghei* immunized rats (Schofield et al. 1987). We have recently found that *P. berghei* infection of HC-04 cells *in vitro* can be limited by the soluble factors released from bystander-activated CTLs, which can be mostly recreated with a combination of TNFα and IFNγ (S. Baer et.al., unpublished). These findings support other *in vitro* studies illustrating a dose- and time-dependent control of *P. yoelii* EEFs by TNFα (Depinay et al. 2011). These studies raise the question of a role for traversed cells in bystander-mediated control of EEFs. We speculated, that if sporozoite antigens shed

during traversal can be presented, recruit and activate specific CTLs to the liver proximal to infected cells, they could play a part in immunity against liver stage infection.

Altogether, sterile protection seen in rodent models is mediated by anti-sporozoite antibodies and T cells targeting liver stage antigens, with a common requirement for CD8+ T cells, but the precise correlates of protection relevant to human infection have yet to be determined. Technical challenges limit the ability to definitively determine which liver stage antigens may be presented by invaded and/or traversed hepatocytes to elicit direct T cell activation.

1.11 Experimental models of malaria Infection: advantages and limitations

Rodent models utilizing multiple mouse and rat strains are exploited for studies of malaria-associated immunity and pathology, despite differences in natural history of disease from that in humans. These experimental models use parasites isolated from their natural host, *Thamnomys rutilans,* which have been adapted for use in laboratory mice or rats, further complicating assessment of pathogenesis and induction of natural host-specific immunity. These host-parasite interactions are incredibly complex, illustrated as differential susceptibilities of rodents from different laboratory strains to challenge with different *Plasmodium* species. For example, BALB/c mice are more susceptible to infection with *P. yoelii*, requiring a lower sporozoites dose to become infected, while C57BL/6 are more susceptible to *P. berghei* (reviewed in Nganou-Makamdop & Sauerwein 2013)) Nonetheless, rodents remain the most convenient animal model of infection and immunity, as compared to non-human primates and humans. Some of the most insightful findings from rodents have defined the dynamics of early events of

infection, from sporozoite inoculation to its destination in the skin, lymph node, or liver (reviewed in (Wykes & Good 2009)

Different genetic backgrounds of inbred mice and *Plasmodium* species are selected as tools to study specific phenomena thought to relate to human malaria infection (reviewed in (Zuzarte-Luis et al. 2014). Briefly, three common species utilized in the laboratory include *P. berghei, P. yoelii yoelii, and P. chabaudi*. *P. berghei* is used to recapitulate a model of cerebral malaria upon infection in C57BL/6 mice. It is useful for the availability of tools for genetic manipulation and high *in vitro* infection rates of human hepatocyte lines (Hollingdale et al. 1983) and primary hepatocytes (Long et al. 1989). *P. yoelii yoelii* is thought to invade and develop most similarly to *P. falciparum in vitro*, sharing the requirement for CD81, unlike *P. berghei*, which does not require CD81 for hepatocyte invasion (Silvie et al. 2003). Finally, *P. chabaudi* is often utilized to study illness that is most similar to that caused by *P. falciparum*, including pathogenesis due to erythrocyte sequestration and antigenic variation. Most recently, the use of several chimeric mice with humanized livers allow monitoring of the development of *P. falciparum* liver stage infection, but in the absence of adaptive immunity (Legrand et al. 2009).

Mice are frequently used in early stages of vaccine testing. However, protective antimalarial immunity in mice has been readily achieved in many vaccine models using a variety of antigens. These studies have rarely translated into success using the same vaccine design in humans, questioning the utility of antigen discovery and validation of vaccine efficacy in mice. Instead, rodent models may have more utility in defining protective immunological correlates, such as the abundant evidence for protection from

CSP-specific CTLs, to inform vaccine design and studying cellular host-pathogen interactions.

Since there is clearly no true small animal model of *P. falciparum* malaria illness (Miller et al. 1994), we often turn to *in vitro* studies, which present their own limitations. *In vitro* studies are hindered by low invasion rate, inefficient EEF development, biological variability in sporozoites production, and often subjective measures of infection. Nonetheless, *in vitro* infection of human cells with *P. falciparum* has been the optimal experimental system for our studies of sporozoites traversal, antigen presentation, and T cell activation.

1.12 Thesis overview

The enormous body of research on malaria in humans done so far is still insufficient to undertake a rationale approach to developing effective vaccines against pre-erythrocytic infection. While liver-stage-specific CD8+ T cells are accepted to be important in protection, it remains uncertain how to induce a strong, long-term effector memory T cell pool that can efficiently home to and reside in the liver. We used two infection models to develop a better understanding of CTL responses to liver-stage infection: *in vitro* infection, closely investigating sporozoite traversal, and memory responses mounted by peripheral blood from healthy adults living in malaria-endemic Mali.

The main goals of the study were to:

a. Determine if liver stage antigen implicated in anti-malarial immunity, circumsporozoite protein, can be processed and presented by hepatocytes traversed by *P. falciparum*
b. Determine if traversed hepatocytes can activate CD8+ T cells in an antigen-specific, MHC-class I-restricted manner
c. Examine humoral and cellular responses against CSP in peripheral blood of naturally exposed adults

1.12.1 Experimental system

We sought to use an *in vitro* model of sporozoite infection and traversal relevant to human infection with *P. falciparum*. We employed two primary tools: human hepatocyte line HC-04 and the 3D7-HTGFP strain of *P. falciparum*, as described in Materials and Methods. HC-04 is the only immortalized cell line shown to support the development of both *P. falciparum* and *P. vivax* EEFs. *P. falciparum* EEF development is difficult to achieve *in vitro* and optimization of such methods were the primary goal of many studies (March et al. 2013; Ng et al. 2014; Tao et al. 2014; Zou et al. 2013; Prudêncio et al. 2011). We routinely observe infection rates, as measured by GFP percentage, of around 0.1% at maximum. However, sporozoites readily traverse through hepatocytes *in vitro*, as we will show in Chapter 2, making traversed cells available for study. For a limited number of experiments, we used cryopreserved human hepatocytes.

Chapter 2. HUMAN HEPATOCYTES TRAVERSED BY *P. FALCIPARUM* SPOROZOITES *IN VITRO* RETAIN CIRCUMSPOROZOITE PROTEIN WITH DYNAMIC SUBCELLULAR LOCALIZATION

This chapter contains material adapted from the manuscript:

Trop S, Dumoulin P, Ma J, Zhang H and J Levitskaya. Traversed hepatocytes retain, process and present *Plasmodium falciparum* circumsporozoite protein for CD8+ T cell recognition.

2.1 Introduction

The liver stage of *Plasmodium* infection is an optimal target of protective vaccines because abrogation of this asymptomatic phase will prevent progression to clinical malaria. Yet, to date, an effective and practical vaccine has not been developed. Since immune responses must be initiated early enough to prevent development of hepatic merozoites, we investigated the immunological implications of the initial interactions between sporozoites and hepatocytes: cell traversal. During sporozoite traversal, or migration in and out of cells, the sporozoite breaches the host plasma membrane, and secretes antigenic proteins, including circumsporozoite protein (CSP).

Sporozoites are covered with CSP, the abundant, immunodominant sporozoite antigen that is transcribed in *P. falciparum* EEFs for five days (Sacci et al. 2006). Since CSP is actively shed apically by sporozoites during gliding motility (Stewart, Michael J., Vanderberg 1988; Stewart & Vanderberg 1991), we hypothesized that it could also be released into hepatocytes during cell traversal. It is conceivable that CSP-specific CD8+ T cell responses may target any cell containing this antigen and expressing MHC class I, including invaded as well as traversed hepatocytes. However, studies on the presence and turnover of CSP in traversed cells not containing a developing parasite have never been

conducted (Hügel et al. 1996). Accordingly, no knowledge on the immunological characteristics of traversed hepatocytes as potential targets for CD8+ T cell responses is currently available.

In this study, we systematically characterized the retention and processing of *P. falciparum* CSP by traversed human hepatocytes. We demonstrated that CSP deposited in traversed cells is long-lived, resides within multiple cellular compartments and serves as a substrate for degradation by several proteolytic machineries which are expected to be presented by both canonical and non-canonical processing pathways resulting in MHC class I presentation.

2.2 Materials and Methods

Cell lines

Human hepatocyte line HC-04 (Sattabongkot et al. 2006) was obtained from ATCC (Manassas, VA) and maintained in "complete medium": IMDM containing 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine (Gibco, Grand Island, NY) and 2.5% fetal bovine serum (FBS) (Corning Cellgro, Manassas, VA). Cryopreserved primary human hepatocytes were obtained from Triangle Research Labs (Research Triangle Park, NC) and maintained in complete medium containing 10% FBS.

Isolation of *P. falciparum* sporozoites

P. falciparum 3D7-HTGFP parasite strain (Talman et al. 2010) was kindly provided by R. E. Sinden (Imperial College, London, UK). Female *Anopheles stephensi* mosquitoes were fed with blood containing *P. falciparum* gametocytes generated in the Insect and Parasite Core Facility (Johns Hopkins Malaria Research Institute, Baltimore,

MD). Mosquitoes were maintained on 10% sucrose containing penicillin, streptomycin and gentamicin for 17 days prior to sporozoite isolation from the thoraces of 800-1000 mosquitoes per experiment as previously described (Ma et al. 2013).

P. falciparum infection and isolation of traversed hepatocytes

HC-04 cells were seeded in 12-well plates at a density of 6.5 x 10^4 cells per well 72 h prior to infection. *P. falciparum* sporozoites were added at 1:1 sporozoite-to-hepatocyte ratio in complete medium containing 0.2 μg/ml of fluoro-emerald, fluoro-ruby or Alexa Fluor 647 conjugated 10,000 MW dextran (Molecular Probes, Grand Island, NY). Alexa Fluor 647 conjugated dextran was used for cell sorting prior to immunofluorescence experiments. In control experiments cytochalasin D (Sigma-Aldrich, St. Louis, MO) treated or untreated sporozoites were incubated on ice for 30 min prior to infection. Plates were centrifuged at 380 x *g* for 5 min without brake. Three hours post-infection, cells were washed three times with PBS containing 10% FBS. Separation of traversed from nontraversed, propidium iodide (PI) negative, hepatocytes was done 3-4 h pi using a MoFlo Cell Sorter (Beckman Coulter, Indianapolis, IN). To assess parasite development, percentages of GFP+PI- HC-04 cells were determined by flow cytometry 72 h pi.

Immunoblotting

Traversed HC-04 cells were isolated by flow-cytometry based sorting 2 h pi, re-seeded and propagated with or without relevant treatment as described above, collected with 0.05% Trypsin-EDTA, washed with PBS, lysed with RIPA buffer (Cell Signaling Technology, Danvers, MA) on ice and 2x Laemmli Buffer (Bio-Rad, Hercules, CA) was added. Sonicated and boiled (95°C, 5 min) cell lysates were resolved on 10% Criterion

Tris-Glycine SDS-PAGE gels (Bio-Rad) and transferred to PVDF membranes (Bio-Rad). Membranes were blocked in 5% non-fat milk in 0.1% Tween-20/PBS (PBST) and incubated with primary antibodies overnight in 5% milk/PBST at 4°C followed by washing and incubation with secondary antibody for 1 h at room temperature. The following antibodies were used: anti-*Pf*CSP clone 2A10 (Zavala et al. 1985b) (kind gift of Dr. F. Zavala, JHMRI), anti-β-actin (Sigma, St. Louis, MO), anti-polyubiquitin (Enzo, Farmingdale, NY), anti-LC3B (Cell Signaling Technology, Danvers, MA) and anti-mouse or anti-rabbit IgG-HRP (GE Healthcare, Piscataway, NJ). Membranes were developed with ECL Prime reagents and exposed to film (GE Healthcare).

Immunofluorescence microscopy

Sorted hepatocytes were propagated on collagen-coated coverslips for 24, 48 or 72 h (NeuVitro, El Monte, CA). Coverslips were washed three times in PBS, fixed for 20 minutes in 4% paraformaldehyde, permeabilized/blocked with 0.1% Triton X-100, 10% goat serum and 1% BSA in PBS and incubated with the following primary antibodies for 1 h at room temperature: anti-CSP clone 2A10 (1 μg/ml) and parasite anti-Hsp70 clone 4C9 (2 μg/ml) kindly provided by F. Zavala (JHMRI, JHU); anti-LAMP1 (Sigma), anti-EEA1, -LC3B, -p62, -Rab7 (Cell Signaling Technology), anti-ubiquitin-protein conjugates (Enzo). Mouse IgG2a or normal rabbit serum (Sigma, St. Louis, MO) were used as relevant isotype control antibodies. After washing with 1% BSA in PBS, coverslips were incubated with secondary goat anti-mouse IgG-Alexa Fluor 594, isotype specific anti-IgG2a Alexa Fluor 488 or anti-IgG1 Alexa Fluor 488, or goat anti-rabbit IgG-Alexa Fluor 488 antibodies (Molecular Probes, Grand Island, NY) and mounted with ProLong Gold containing DAPI (Molecular Probes). Widefield fluorescence microscopy

was performed on a Nikon 90*i* upright microscope and images were acquired with a Hamamatsu ORCA 1394 camera and Volocity software (Perkin Elmer, Akron, OH). Patterns of CSP were classified visually as either punctate, contained within a single large aggregate, nuclear, or cytosolic. Overlap coefficients of CSP and markers of cellular compartments were calculated in Volocity as the percentages of the surface area of CSP that co-localized with the indicated cellular marker using automated thresholding to identify objects. Overlaid micrographs of co-localized points, as determined by same thresholds, were generated using the RG2B plugin in ImageJ (Rasband 2012).

Antibody staining and flow cytometry

For intracellular detection of *P. falciparum* CSP and Hsp70, monoclonal antibodies 2A10 and 4C9 were directly conjugated to fluorophores using Zenon labeling kit for IgG2a and IgG1 following manufacturer's instructions (Molecular Probes, Grand Island, NY). Cells were fixed and permeabilized prior to intracellular staining as described above. Data were analyzed in FlowJo software (TreeStar, Ashland, OR).

Treatment with proteolytic inhibitors

Sorted, traversed HC-04 cells were treated at different time points pi with chemical inhibitors: proteasomal inhibitor lactacystin (20 μM, Sigma); inhibitor of lysosomal acidification, chloroquine (100 μM, Sigma); autophagy inhibitor 10 μM spautin-1 (Cellagen Technology, San Diego, CA) for 16 h prior to sample collection.

Statistical methods

Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA). Bar graphs display mean ±SE. One-way ANOVA was used to

compare more than two groups. Contingency tables with Chi-square or Fisher's Exact Test were used to compare frequency distribution of patterns of CSP at different times or following proteolytic inhibitor treatment.

2.3 Results

2.3.1 Flow-cytometry based detection and sorting of traversed cells

Traversed hepatocytes were identified by a flow-cytometry based membrane wounding assay adapted from previously described methods (Prudêncio et al. 2008). *P. falciparum* sporozoites were added to a monolayer of HC-04 cells in medium containing 10,000 MW dextran conjugated to fluorescein, tetramethylrhodamine, or Alexa Fluor 647. At 2 h pi, there was a small background (0.307%) of dextran-positive cells noninfected cultures, 27.8% dextran uptake in *Pf*-infected cultures, and a near-complete return to background levels of dextran-positivity (2.45%) with the addition of Cytochalasin D, a chemical inhibitor of actin polymerization (Figure 2.1). We also used this strategy to separate nontraversed (dextran-, PI-) from traversed (dextran+, PI-) cells by fluorescence activated cell sorting (Figure 2.2).

Though it is frequently used, questions remain regarding the interpretation of this assay, such as if the dextran enters upon sporozoite entry or exit, the significance of the range of fluorescence intensities observed, and whether or not dextran is taken up during invasion as well. It is thought that as the sporozoite breaches host the plasma membrane as it enters for traversal, producing a small hole in which the fluorescent dextran can enter, thus the percent of cells that take up dextran reflects the percent of cells that were traversed (Mota et al. 2001). An alternative assay quantifies traversal by measuring the release of calcein pre-loaded into cells, providing information about the frequency of cell

wounding for an entire population (Coppi et al. 2007). While we speculate that the fluorescence intensity of dextran could be an indicator of the number of times a single cell was traversed, this has yet to be demonstrated. It is also unclear if productive sporozoite invasion leads to dextran uptake or not. Using HepG2/CD81 and HepG2, which are and are not permissive to productive invasion with *P. yoelii*, respectively, dextran was found in cells with sporozoites that had entered the cell without productive PV formation, but not those with PVs (Silvie et al. 2006). The investigators concluded that productive invasion does not result in dextran uptake. While *P. yoelii* is thought to be the most relevant surrogate rodent parasite for *P. falciparum* invasion of hepatocytes (March et al. 2013), it has yet to be demonstrated if *P. falciparum* invasion is accompanied by dextran-positivity. Due to the technical difficulty of distinguishing cells that have been only invaded from cells that have been invaded as well as traversed, we were unable to determine if *P. falciparum* invasion leads to dextran uptake.

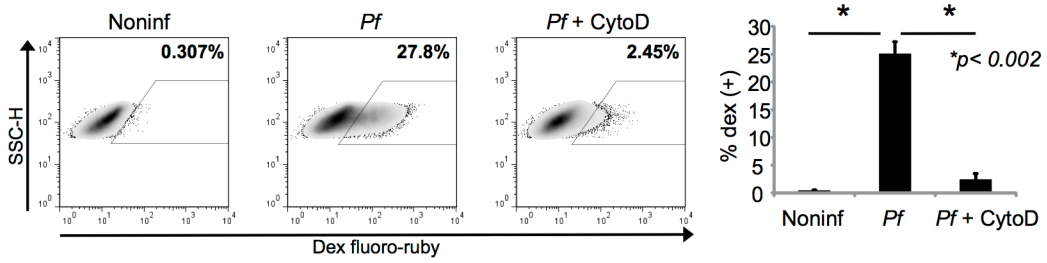


Figure 2-1. Flow cytometric detection of sporozoite traversal

Representative example of flow cytometry-based detection of traversed cells. Cytochalasin D, an inhibitor of actin polymerization, was used to demonstrate that dextran uptake required active sporozoite motility and was not due to nonspecific wounding of the hepatocyte plasma membranes by other components present in sporozoite preparations. Bar graph summarizes same experiment in triplicate. * = significance following t-tests corrected for multiple testing.

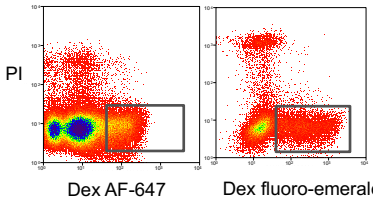


Figure 2-2. Fluorescence activated cell sorting of traversed cells

Viable traversed cells were isolated by flow cytometry based cell sorting using dextran conjugated to different fluorophores in independent experiments. Gating is based on specific dextran uptake as shown in (Figure 2.1) in the viable, PI-negative cell population following exclusion of cell debris and cell "doublets" (data not shown).

2.3.2 Traversed cells retain immunodominant antigen CSP in the absence of detectable EEFs

Since sporozoites shed CSP during gliding motility, we asked whether CSP was shed into host cells during traversal. We looked for the presence of CSP by antibody-based methods using monoclonal antibody 2A10, which recognizes the central repeat region.

We analyzed HC-04 cells infected with *P. falciparum* in the presence of dextran-tetramethylrhodamine for the presence of CSP. A representative experiment is shown in Figure 2.3. Four hours pi, we detected CSP in 38.5% of dextran-positive cells after fixation and permeabilization, and in 9.05% of dextran-negative cells (Fig 2.3). We speculate that factors such as the amount of time spent by a sporozoite in a cell, and how large an opening it formed in the host plasma membrane, affected how much dextran and CSP were deposited.

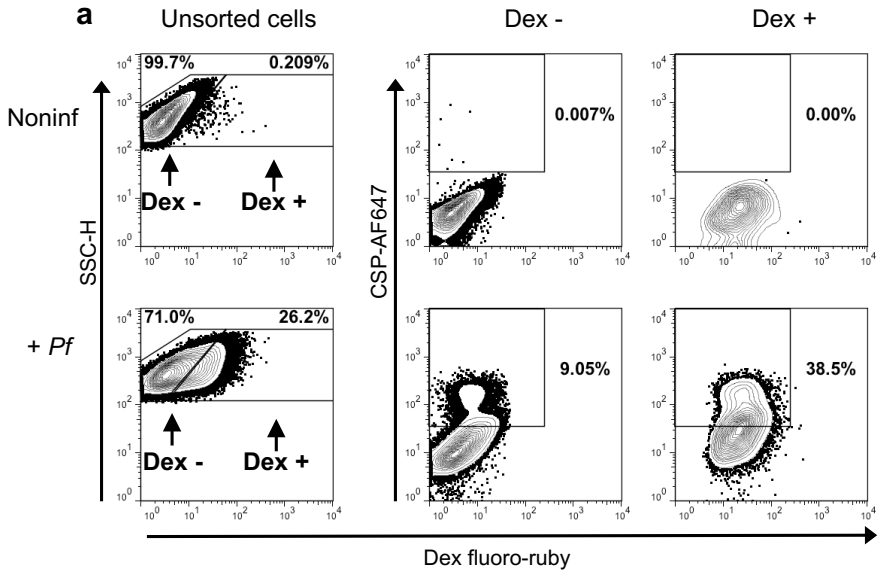


Figure 2-3. Flow-cytometric detection of CSP in traversed cells.

CSP is detected in traversed (Dex+) cells by intracellular staining and flow cytometry 4 h pi. CSP-Alexa Fluor 647 staining is indicated within Dex- and Dex+ populations in unsorted, noninfected (top) and *Pf*-infected (bottom) cultures. Noninfected cultures incubated with dextran show low levels of spontaneous dextran uptake and absence of CSP-specific fluorescence.

Visualization of intracellular CSP, as opposed to surface CSP, by microscopy has long been used to identify sporozoite-invaded cells (Renia et al. 1988). This method has recently been adapted as the basis for a flow cytometric determination of invasion as well

(Kaushansky et al. 2012). In the technique used by Kaushansky and colleagues, 2% of fixed, permeabilized HC-04 cells were CSP-positive. The investigators described this as a 2% invasion rate, which significantly exceeds invasion efficiencies previously reported by many other investigators and our own findings (Dumoulin *et. al.,* submitted): 0.066% for HC-04 cells, (Sattabongkot et al. 2006); 0.09% for HHS-102 cells, (Karnasuta et al. 1995); and only slightly higher in primary human hepatocytes, 0.13%, (Mazier et al. 1985). Thus, we sought to validate the proposed method (Kaushansky et al. 2012) by introducing additional parasite-specific markers. First, we stained live cells from *P. falciparum* – infected HC-04 cultures with 2A10 antibody conjugated to Alexa Fluor 488, to identify cells that had CSP on the surface. In this experiment, 14% of infected cultures retained dextran at 24h pi (Figure 2.4a). Next, we fixed and permeabilized these cells, and stained with 2A10-Alexa Fluor 647, to stain all CSP, including intracellular and surface, if any. In this representative experiment, 15% of cells were CSP-Alexa Fluor 647 positive, and only 0.036% were CSP-Alexa Fluor 488 positive (Figure 2.4b), indicating that nearly all CSP was intracellular, and not on the surface of hepatocytes. Surface CSP is thought to be found on non-invaded cells over which sporozoites shed CSP during gliding motility.

We then performed double staining of infected cultures with antibodies against both parasite-specific CSP and Hsp70. Only 0.099% of all hepatocytes were CSP+Hsp70+ (Figure 2.4c). We propose CSP+Hsp70+ to be a more accurate measure of the percent of parasite-invaded cells than the detection of intracellular CSP alone, which reflects CSP shed in hepatocytes during sporozoite traversal (CSP+Hsp70-).

We address a technical limitation of this 3-color staining protocol. Alexa Fluor 488 is emitted in the same fluorescent channel as parasite-expressed GFP. However, we observe that GFP expression in this parasite strain is weak and difficult to monitor in sporozoites, but increases with parasite replication. The earliest time point that we routinely observe parasite-encoded GFP by flow cytometry is 48 h pi. Therefore, the fluorescence signal emitted by cells stained with anti-CSP-Alexa Fluor 488 is most likely surface CSP, and not GFP.

Altogether, we showed dextran-positive, traversed cells are enriched for CSP versus dextran-negative, non-traversed cells, as expected, since CSP is shed during traversal (Stewart, Michael J., Vanderberg 1988). Additionally, the distinction between CSP detected on the hepatocyte surface and detected intracellularly is insufficient to identify invaded cells.

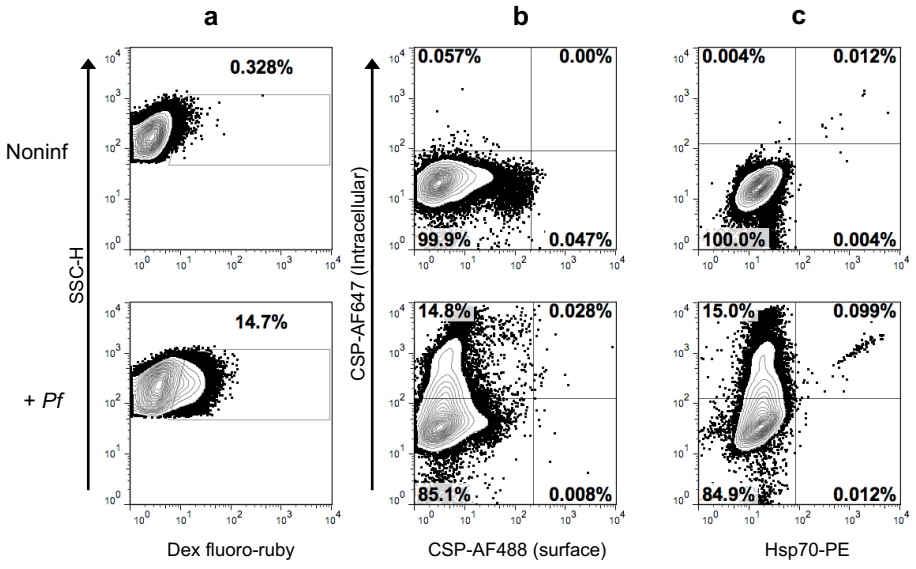


Figure 2-4. Co-detection of CSP and Hsp70 in traversed cells.

CSP retained by traversed cells 24 h pi is almost entirely intracellular. Upper panels - noninfected, lower panels – *Pf* infected cultures. Traversed cells gated as dextran-positive in **(a)** have detectable intracellular, but not surface CSP **(b).** CSP-positive HC-04 cells are

predominantly *Pf*Hsp70-negative **(c).** Intracellular detection of surface and intracellular parasite antigens is described in Materials and Methods.

2.3.3 Traversed cells remain viable and proliferate *in vitro*

Since the hepatocyte plasma membrane is breached during traversal, it would not be surprising if these cells had poor viability, or deteriorated soon after. However, dextran+ HC-04 excluded cell impermeant dye propidium iodide (PI) (Figure 2.2), indicating they were viable at the time of analysis. Sorted, traversed cells proliferate at an equal rate to nontraversed cells (data not shown), consistent with other reports that *P. berghei* infected and traversed HepG2 cells do not show signs of stress by the formation of stress granules (Hanson & Mair 2014). We hypothesized that the viability of traversed cells supports the possibility that they could present sporozoite antigen, namely CSP, for the duration of EEF development.

2.3.4 Traversed cells retain CSP for at least 6 days pi *in vitro*

After sorting traversed and nontraversed cells by FACS, we propagated them *in vitro* and collected cells daily to assess the amount of CSP retained. Cells were distributed uniformly into 24-well plates immediately following sorting (6 h pi), and one well was collected at each indicated time point. The same proportion of cell lysate obtained from cells recovered from each well was loaded onto SDS-PAGE gels. We observed that CSP was still detectable by immunoblot 6 days pi, the latest time point monitored (Figure 2.5a). CSP detected in traversed cell lysate migrated at the same size as sporozoite lysate, and in between cells transiently transfected to express the full length and N-terminal cleave products of CSP (Figure 2.5b).

We identified traversed cells as a source of at least one sporozoite- and liver-stage antigen, CSP. Other parasite proteins have been found in trails of gliding sporozoites, and we speculate they may be deposited in traversed cells as well (Mota et al. 2002). As CSP-specific CD8+ T cells are important in the killing of infected hepatocytes, this raises the possibility for presentation of CSP-derived peptides by traversed cells to recruit and activate T cells in close proximity to infected cells, the desired target of cytotoxicity. We proceeded to further analyze the duration of CSP retention within traversed cells, its subcellular localization, and the proteolytic systems involved in its degradation.

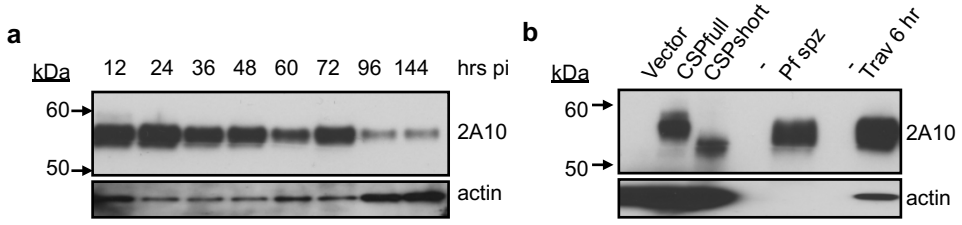


Figure 2-5. CSP detection in traversed cells over 6 days

Sorted traversed cells were re-seeded, propagated and analyzed for presence of CSP by western blot at different time points pi. Migration of CSP retained in traversed hepatocytes on SDS-PAGE gels. **(a)** Time kinetic of CSP retention in traversed hepatocytes. Equal numbers of traversed cells sorted 4 h pi were re-seeded into each well of 24-well plate. Traversed cells were viable and proliferated until time of sample collection. Therefore, samples used for western blotting correspond to equal proportions of the total cellular content obtained from the individual well collected at indicated time point. Data from one representative experiment out of 3 performed are shown. **(b)** HC-04 cells ectopically expressing *P. falciparum* 3D7 CSP in its uncleaved (CSPfull) or mature N-terminal cleavage (CSPshort) form, as well as Pf sporozoite lysate were used as controls.

2.3.5 Retained CSP is detected in six different patterns of subcellular localization in traversed hepatocytes

Since HC-04 cells expressed MHC class I, but not class II, on the cell surface, we next sought to determine if the CSP found in traversed cells resided in a compartment accessible to components of the MHC class I processing machinery. It is well established that cytoplasmic and nuclear proteins are the major source of peptide epitopes presented by MHC class I, whereas proteins residing in vacuolar endosomal/lysosomal compartments are usually presented on MHC class II (reviewed in Vyas et al. 2008). We fixed, permeabilized and stained sorted, traversed cells at 24, 48, and 72h pi with anti-CSP, and visualized cells by fluorescence-widefield microscopy. We found varied subcellular localization of CSP in traversed cells, emerging as 6 distinct patterns of antibody-based staining (Figure 2.6A):

Pattern 1) Homogenous puncta: similar sized puncta spread randomly throughout the cell

Pattern 2) Heterogeneous puncta: variable size and/or focused localization

Pattern 3) Single intracellular aggregate, “intracellular CSP body”: generally located perinuclear

Pattern 4) Single extracellular aggregate tethered to plasma membrane, “extracellular CSP body”

Pattern 5) Nuclear

Pattern 6) Cytosolic

We performed experiments in nonsorted, infected cultures of cryopreserved primary human hepatocytes and observed similar patterns, validating the observations in HC-04 cells (Fig 2.6B).

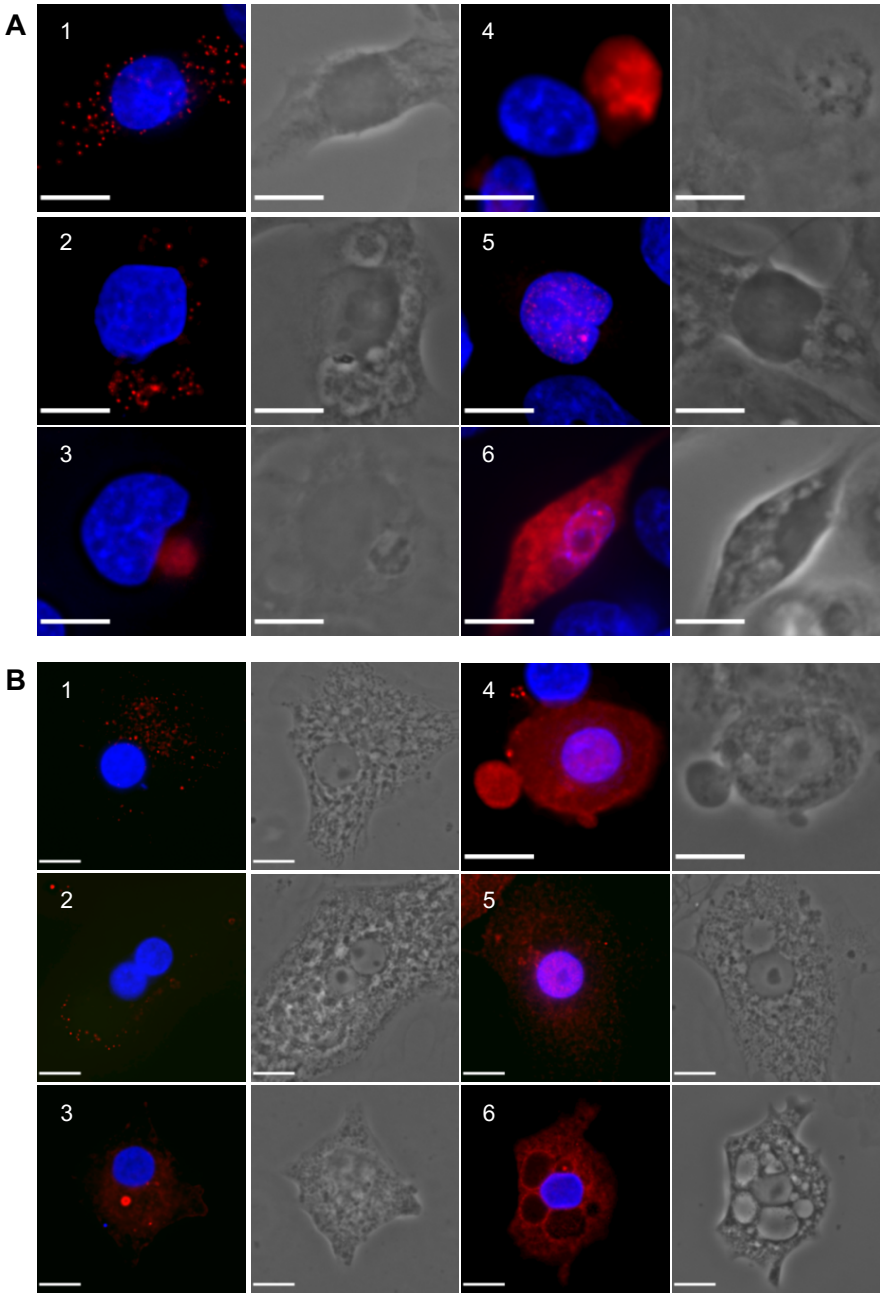


Figure 2-6. Subcellular localization of CSP in traversed hepatocytes.

Sorted, traversed cells were propagated on coverslips from 6 to 72 h pi. Immunofluorescence detection of CSP was performed at 24, 48 and 72 h pi. Representative images are derived from a total of eight independent experiments, 19 cover slips and 174 individual cells. Each experiment utilized separate preparations of infected mosquitoes, isolation of sporozoites, cell sorting and immunostaining. **(A)** *P. falciparum* CSP is found in traversed HC-04 hepatocytes in six distinct staining patterns: (1) homogeneous puncta; (2) heterogeneous puncta; (3) large intracellular aggregates or "intracellular CSP bodies"; (4) large extracellular aggregates or "extracellular CSP bodies"; (5) nuclear; (6) diffuse cytoplasmic. **(B)** Six patterns of *P. falciparum* CSP retained in primary human hepatocytes are similar to those in HC-04 cells. Scale bars = 10 μM.

2.3.6 CSP bodies are distinct from developing EEFs

Patterns 3 and 4 (Figure 2.6A) were large foci containing CSP that appear to be membrane-bound, with morphology similar to that of a parasitophorous vacuole (PV). Thus, we sought to determine if these structures contained developing EEFs or remnants of dead or dying parasites. We looked for localization of DAPI and Hsp70 to the CSP-containing compartment, and found both to be absent (Figure 2.7). Positive control staining in Figures 2.7A,B show that a sporozoite and early PV contain DAPI and Hsp70, whereas the Pattern 4 "CSP body" in Fig. 2.7C only shows CSP-specific staining. Therefore, "CSP bodies" do not contain developing parasites. However, we cannot formally exclude the possibility that this compartment contains remnants of a dead EEF, and the CSP has a longer half-life than Hsp70 or the parasite DNA. Regardless of whether the "CSP body" originated from an intact EEF or not, the presence of CSP as a single parasite antigen in this pattern is of interest due to its potential as a source for presentation CSP-specific CTLs.

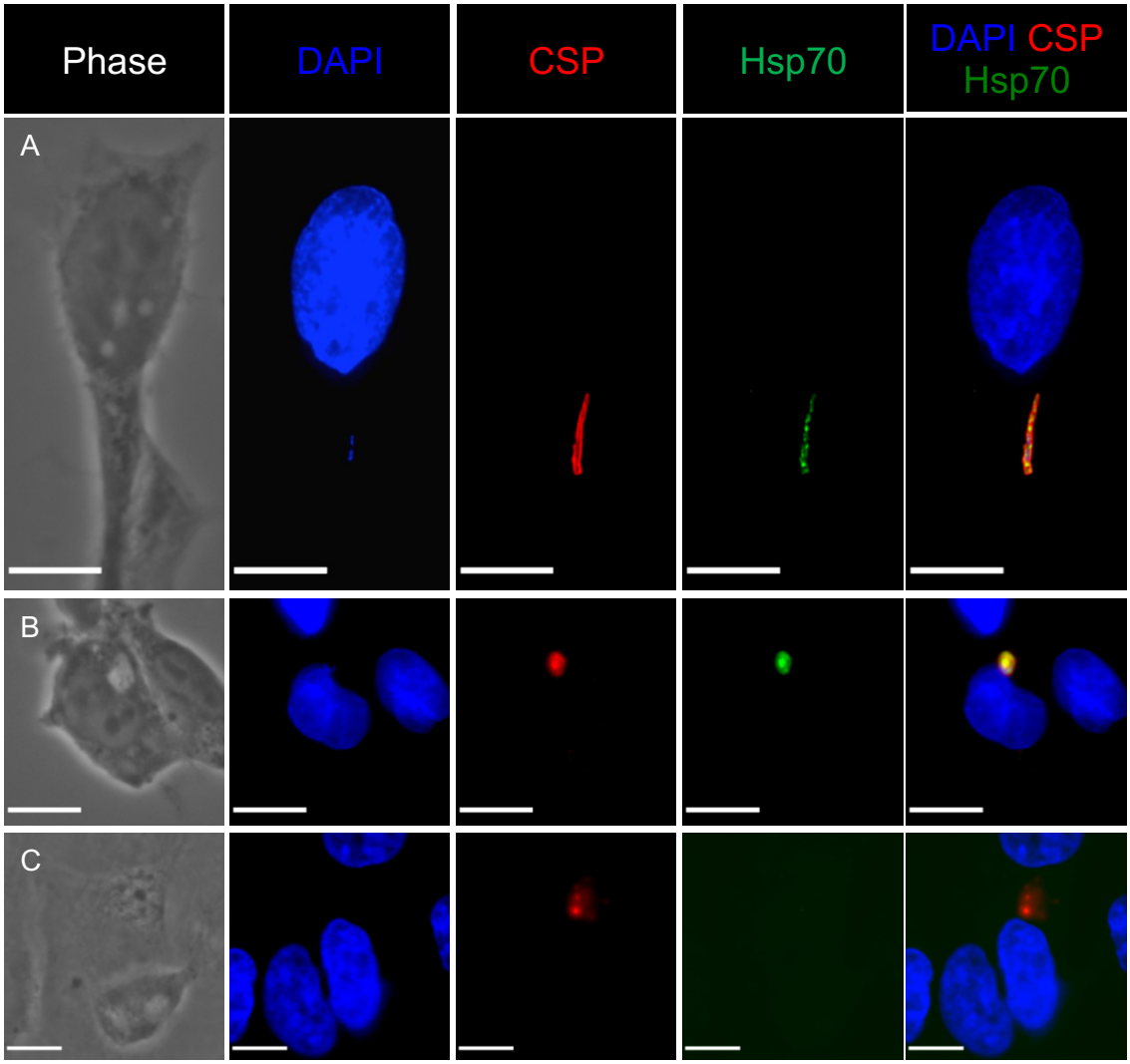


Figure 2-7. "CSP bodies" are distinct from P. falciparum EEFs developing in human hepatocytes.

Dense intracellular compartments containing CSP are devoid of markers characteristic for *P. falciparum*, DAPI and PfHsp70. Whereas parasites with morphology of sporozoite **(A),** and trophozoite **(B)** were stained positive for DAPI and PfHsp70, these markers were not detected in subcellular compartment containing CSP-bodies (Pattern 3) **(C).** Blue = DAPI, red = anti-*Pf*CSP 2A10, green = anti-*Pf*Hsp70 4C9. Scale bars = 10 μM.

2.3.7 Nuclear CSP is detected in the absence of an intranuclear EEF

Reportedly, *P. falciparum* and *P. yoelii* EEFs can partially develop within the nucleus of non-permissive cell type HepG2 (Silvie et al. 2006), so we determined if the nuclear CSP (Pattern 5) we detected in traversed cells was, likewise, an early EEF. We

observed that CSP in the nucleus of traversed cells was distinct from intranuclear EEFs, as shown in Figure 2.8. We detected in the same field of view (1) a cell with a cytoplasmic sporozoite, which had detectable CSP, DAPI, and Hsp70 and (2) a cell with CSP in the nucleus, without Hsp70. Nuclear antigens are generally processed by the proteasome for MHC class I presentation (reviewed in (Neefjes et al. 2011)). Without evidence of sequestration by a PV, we speculated that nuclear CSP in traversed cells is likely available for presentation on MHC I.

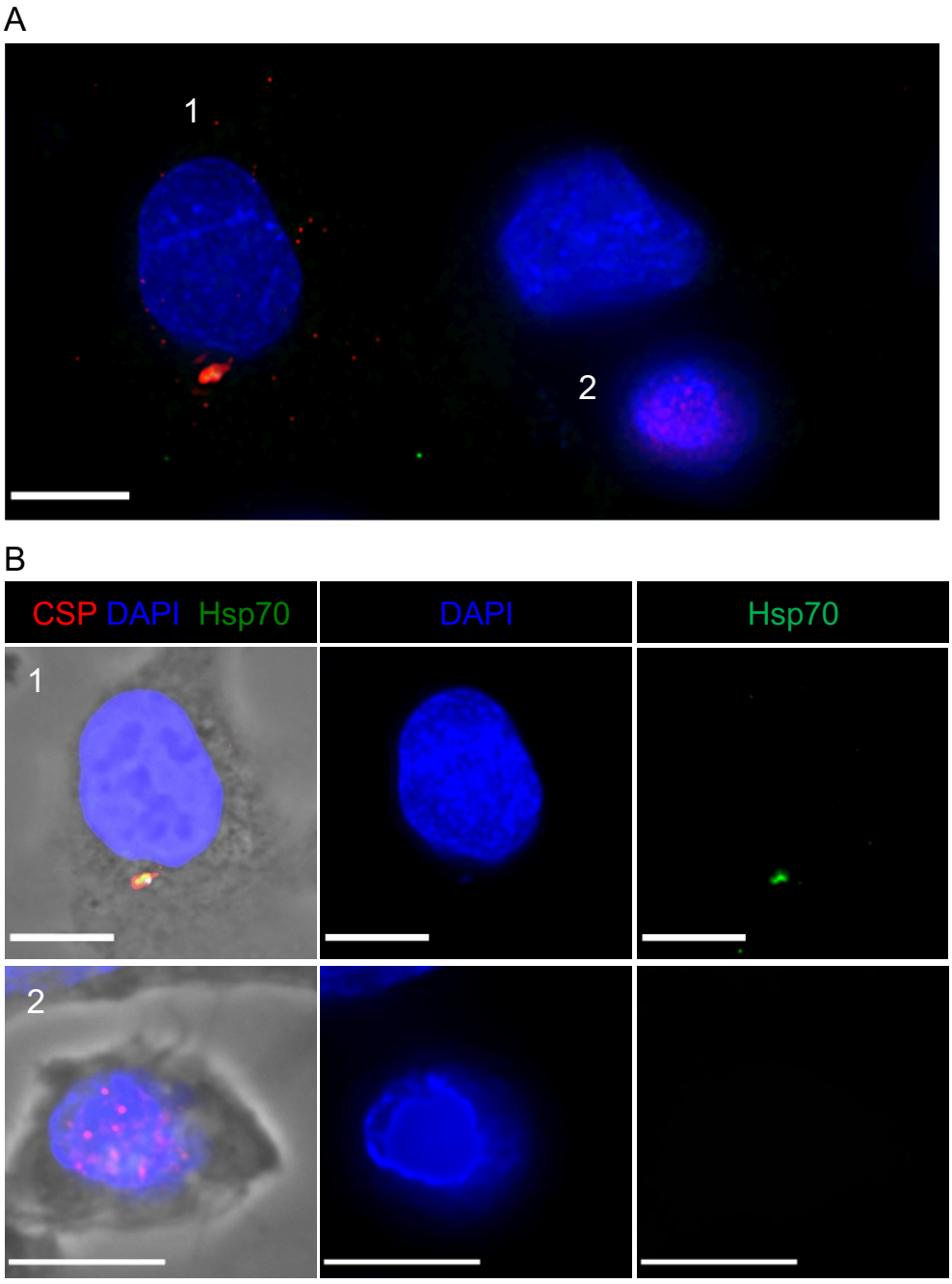


Figure 2-8. Nuclear pattern of CSP in traversed cells is not associated with nuclear EEFs.

CSP in hepatocyte nucleus (Pattern 5) is not accompanied by *Pf*Hsp70 detection. **(A)** Top micrograph shows two CSP-containing HC-04 cells found in same field of unsorted hepatocyte cultures. **(B)** Below, individual panels show detectable DAPI and *Pf*Hsp70 in cell 1 but not in cell 2, which contains CSP in the nucleus. Blue = DAPI, red = anti-*Pf*CSP 2A10, green = anti-*Pf*Hsp70 4C9. Scale bars = 10 μM.

2.3.8 Subcellular localization of CSP fluxes over time, with CSP becoming increasingly aggregated

When analyzing the localization of CSP in traversed HC-04 cells over time, a clear trend appeared: CSP tended to localize to increasingly large aggregates (Figure 2.9). CSP was predominantly found (40%) in homogenous puncta (Pattern 1) 24h pi. The frequency of this pattern declined to about 10% 48h pi and 2% at 72h pi. This decrease was paralleled by a rise in the frequency of cells with CSP in heterogeneous puncta (Pattern 2). Heterogeneous puncta were also prominent 24 h pi, increased slightly at 48 h pi, and returned to initial levels 72 h pi. Remarkably, both intra- and extracellular "CSP bodies" accumulated over time and were found in 12% and 15%, respectively, of all CSP-containing traversed cells at 72 h pi. Similar to CSP bodies, the frequency of cells with diffuse cytoplasmic CSP elevated from 2% at 24 h pi to 19% at 48 h, remaining stable through 72 hours. Finally, the nuclear pattern of CSP (Pattern 5), found in approximately 8% of traversed cells at 24 h pi, rapidly decreased and was undetectable at 72 h pi. Thus, at least two patterns of CSP retention compatible with classical MHC class I antigen processing and presentation, cytoplasmic and nuclear, were found in nearly one third of CSP-containing traversed hepatocytes at 72 h pi, though the nature of other intracellular compartments containing the remaining CSP moieties was still undefined.

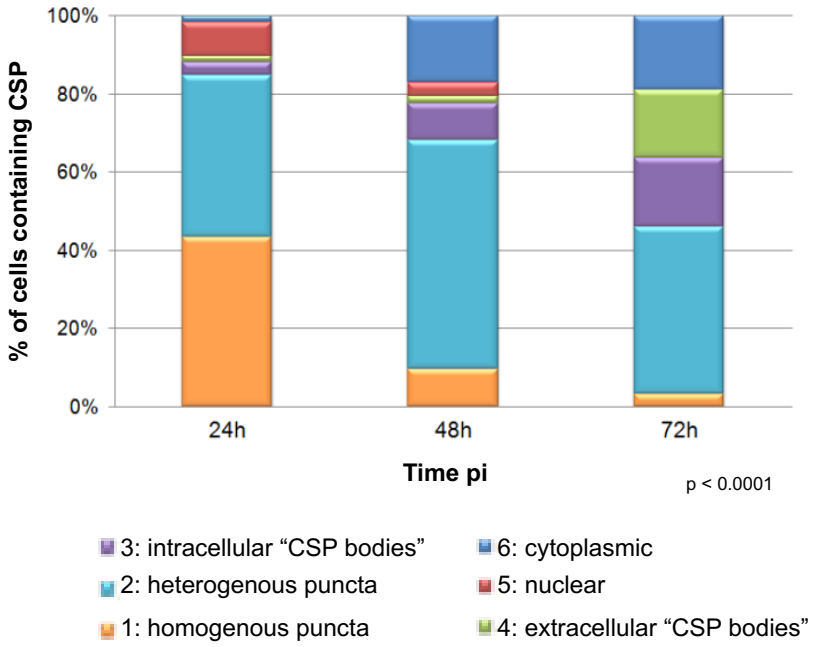


Figure 2-9. Dynamic changes of CSP subcellular localization in traversed HC-04.

Percentages of cells bearing each of the six CSP patterns from all CSP-containing traversed cells analyzed at 24, 48 or 72 h pi., $p < 0.0001$ for comparison of the frequency distribution of CSP patterns across three time points by Chi-square and Fisher's Test

2.3.9 Proposed progression of CSP aggregation

Drawing from the alteration of frequency with which each pattern is found over time, we speculated that CSP aggregates as illustrated in Figure 2.10. It appears that CSP transitions from Pattern 1 to 2 to 3 to 4, becoming increasingly aggregated as the cell deals with CSP as a misfolded protein, eventually expulsing it. The primary question of whether or not each of these pools of CSP is accessible to the proteasome for loading onto MHC class complexes was still unanswered. To try to understand the processes causing this progression, we co-stained traversed cells with antibodies specific for CSP and several markers for organelles associated with proteolytic processing.

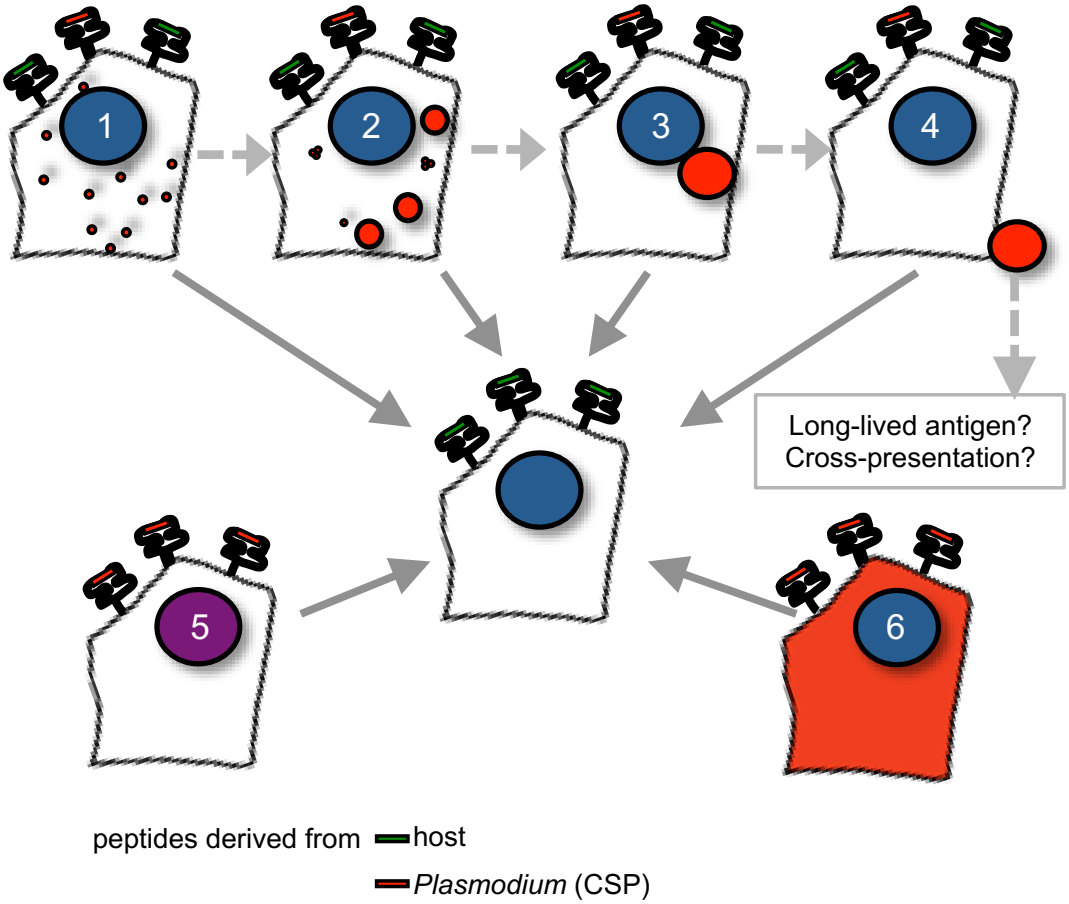


Figure 2-10. Proposed dynamic changes in patterns of retention and MHC class I presentation of CSP in hepatocytes traversed by *P. falciparum* sporozoites.

Schematic of CSP Patterns 1 – 6. Red = CSP and CSP-derived MHC class I peptides. Green = class I peptides derived from self-antigens. Arrows represent proposed transition through patterns as CSP aggregates. We speculate that due to the complex, repetitive structure of CSP, it is recognized by the host cell as a misfolded protein, actively diverted into inclusion bodies in the lysosomal-autophagosomal pathway and either degraded intracellular or expulsed in the form of large aggregates. We further speculate that whereas "vesicular" Patterns 1, 2, and 3 may allow for at least some presentation of CSP epitopes on MHC class I via nonclassical, TAP-independent processing and presentation pathway, as previously described for other antigens (Del Val et al. 2011). Patterns 5 and 6 will serve as a major source for CSP-derived MHC class I-restricted epitopes. We infer that Pattern 4 does not permit presentation of CSP by traversed hepatocytes, as this protein is no longer processed by the cell. Instead, we propose that these extracellular CSP aggregates may constitute a long-lived source of antigen for cross presentation by liver-resident professional antigen presenting cells, thus contributing to the induction and/or maintenance of parasite-specific immune responses.

2.3.10 Subcellular compartments containing CSP contain ubiquitin, lysosomal and autophagic molecular markers

We co-stained sorted, traversed HC-04 cells with a panel of antibodies specific for compartments associated with protein processing: lysosomal-associated membrane protein 1 (LAMP1), LC3B (a component of the autophagosomal membrane) (reviewed in (Barth et al. 2010)), p62 (involved in targeting ubiquitinylated substrates to the autophagosome for clearance of protein aggregates) (Pankiv et al. 2007), polyubiquitin (indicating proteins tagged for proteasomal or autophagosomal degradation such as aggregates of defective ribosomal products or other misfolded proteins (Wenger et al. 2012)), early endosome marker EEA1 and late endosome marker Rab7. Representative images and the percent co-occurrence for each molecular marker within the selected CSP pattern are shown in Figure 2.11.

CSP in homogeneous puncta (Pattern 1) co-localized frequently with polyubiquitin (38%) and Rab7 (39%), less frequently with LAMP1 (15%), LC3B (18%), and p62 (22%), but minimal co-occurrence was observed with EEA1, indicating that these CSP moieties are associated with lysosomal and autophagosomal compartments, and not early endosomes (Figure 2.11A).

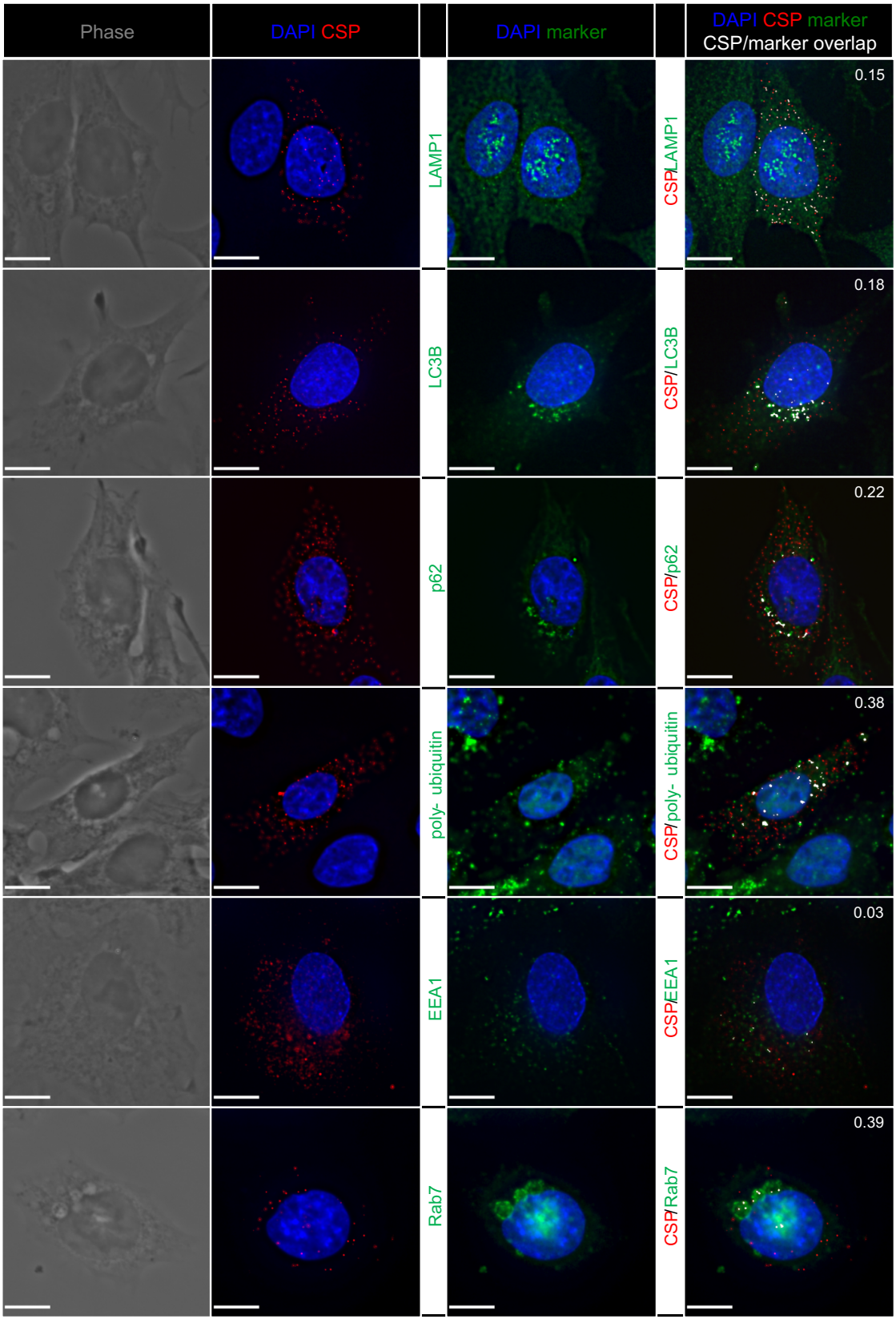
CSP in heterogeneous puncta (Pattern 2) was almost completely associated with polyubiquitin (98%) and LC3B (73%) within vacuoles visible by phase contrast microscopy, suggesting its interaction with a different compartment than homogenous puncta (Figure 2.11B). Less frequently, Pattern 2 CSP co-localized with LAMP1 (15%) and Rab7 (33%).

Similarly to CSP in Pattern 2, a substantial portion of intracellular CSP bodies (Pattern 3) co-localized with LC3B (34%) (Figure 2.11C). However, dissimilar to punctate CSP, CSP bodies were not associated with p62. This suggests that autophagy-related compartments containing CSP in Pattern 2 have a distinct molecular composition from those characterized as Pattern 3. This observation was further supported by the higher co-localization of intracellular CSP bodies with Rab7 (94%). Rab7 is important in the formation of large, late autophagic compartments (Jäger et al. 2004), fitting our proposed model of a sequential progression from small puncta to large, aggregate CSP bodies.

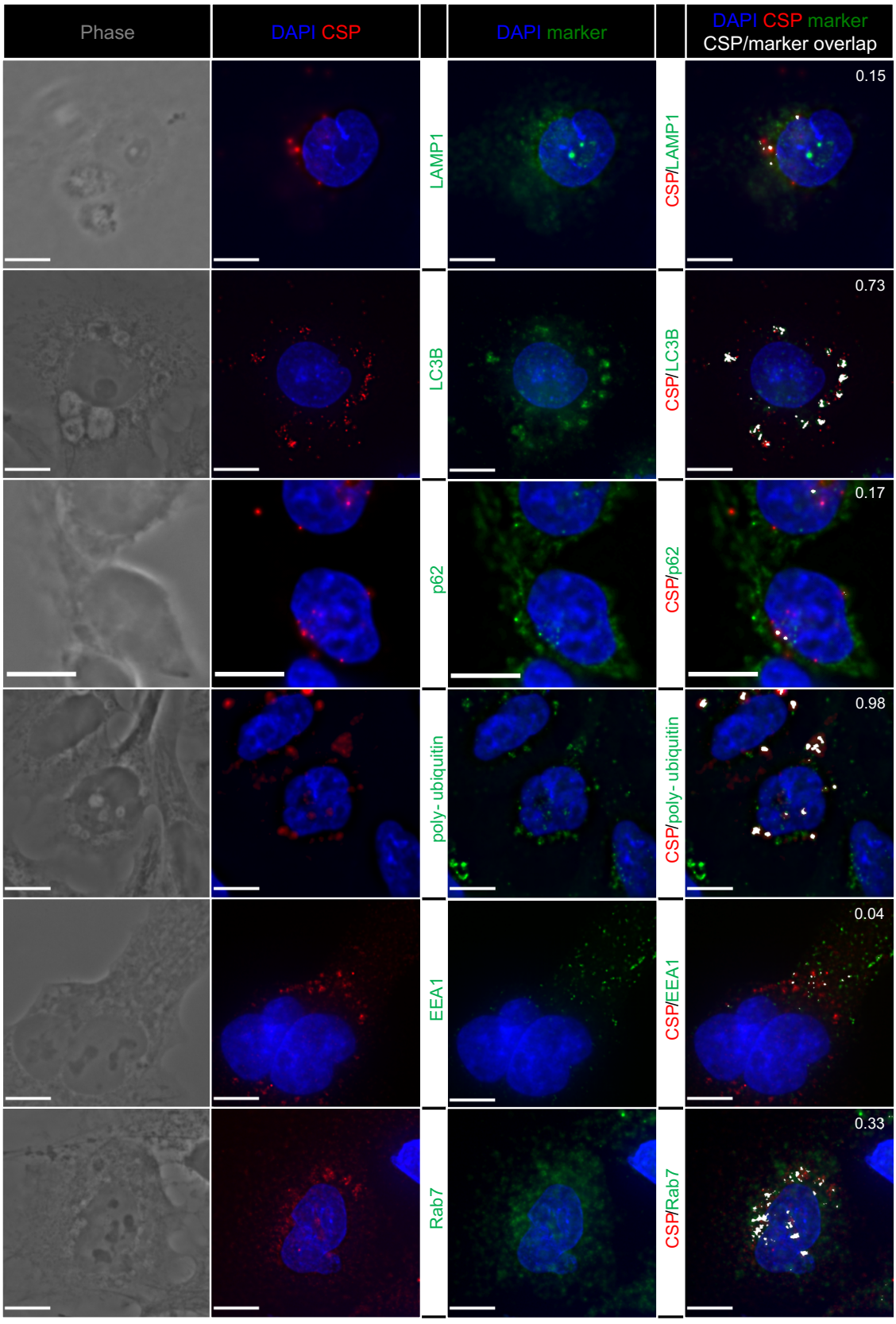
When CSP bodies appeared to transition to a dense, membrane-bound structure externally tethered to a host cell visible by phase contrast (Pattern 4), the molecular composition of the CSP-containing compartment also shifted (Figure 2.11D). Though extracellular CSP bodies still contained the late endosome marker Rab7, these aggregates largely lost their association with LAMP1. Because Pattern 4 occurred relatively infrequently, we were unable to detect it in any p62- or LC3B- stained samples. Patterns 5 and 6 were not analyzed in this manner because none of these molecular markers possess a diffuse cytoplasmic or nuclear distribution.

Altogether, our data demonstrate that the close association of CSP with these organellar markers shifted along with the dynamic localization patterns of CSP. We speculated that CSP retained in each compartment might have variable susceptibility to distinct cellular proteolytic systems.

A


Phase
DAPI CSP
DAPI marker
DAPI CSP marker
CSP/marker overlap
LAMP1
CSP/LAMP1
0.15
LC3B
CSP/LC3B
0.18
p62
CSP/p62
0.22
poly- ubiquitin
CSP/poly- ubiquitin
0.38
EEA1
CSP/EEA1
0.03
Rab7
CSP/Rab7
0.39

B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DAPI marker
DAPI CSP marker
CSP/marker overlap
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CSP/LAMP1
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LC3B
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0.73
p62
CSP/p62
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EEA1
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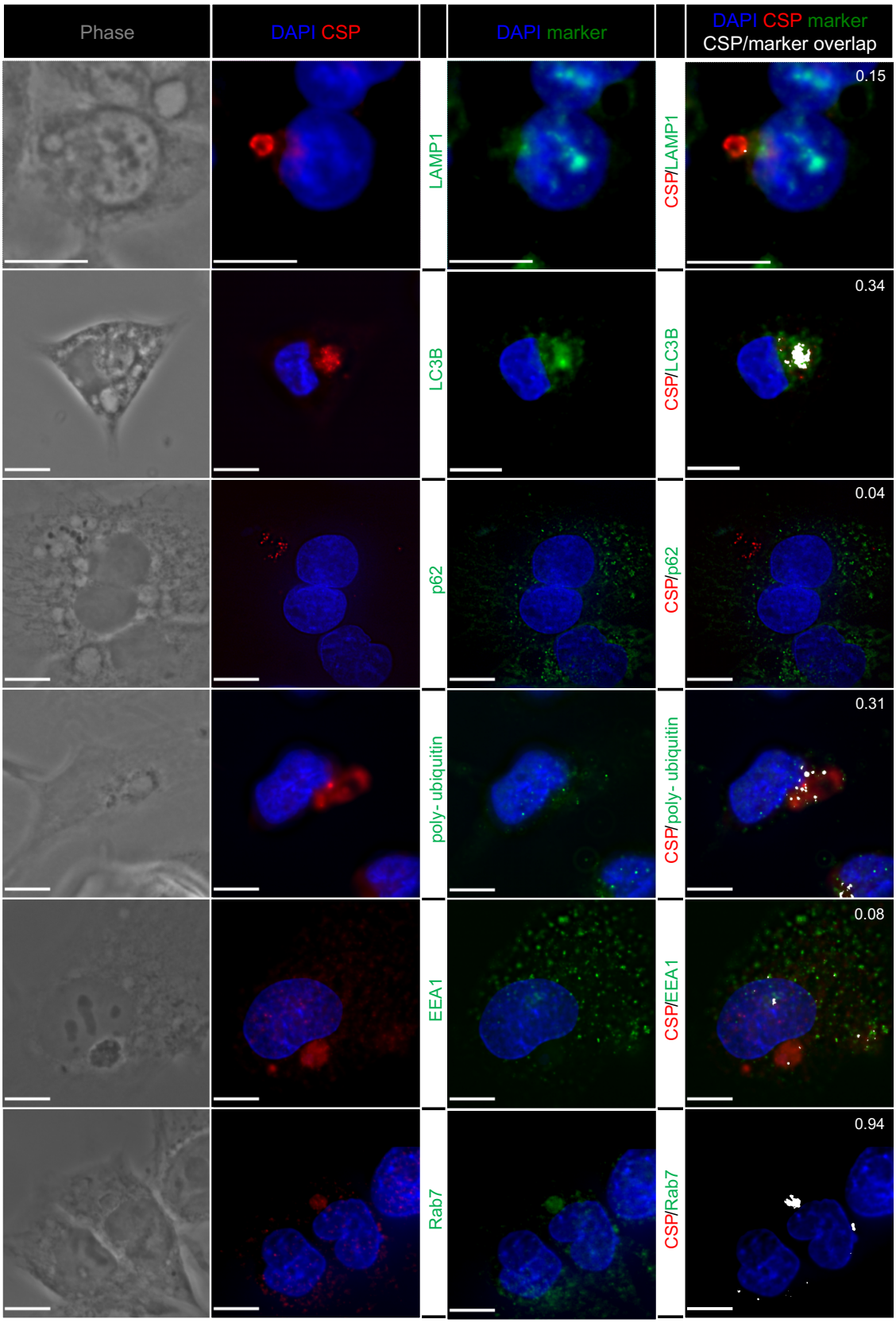
0.04
Rab7
CSP/Rab7
0.33

c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DAPI marker
DAPI CSP marker
CSP/marker overlap
LAMP1
CSP/LAMP1
0.15
LC3B
CSP/LC3B
0.34
p62
CSP/p62
0.04
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EEA1
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0.08
Rab7
CSP/Rab7
0.94

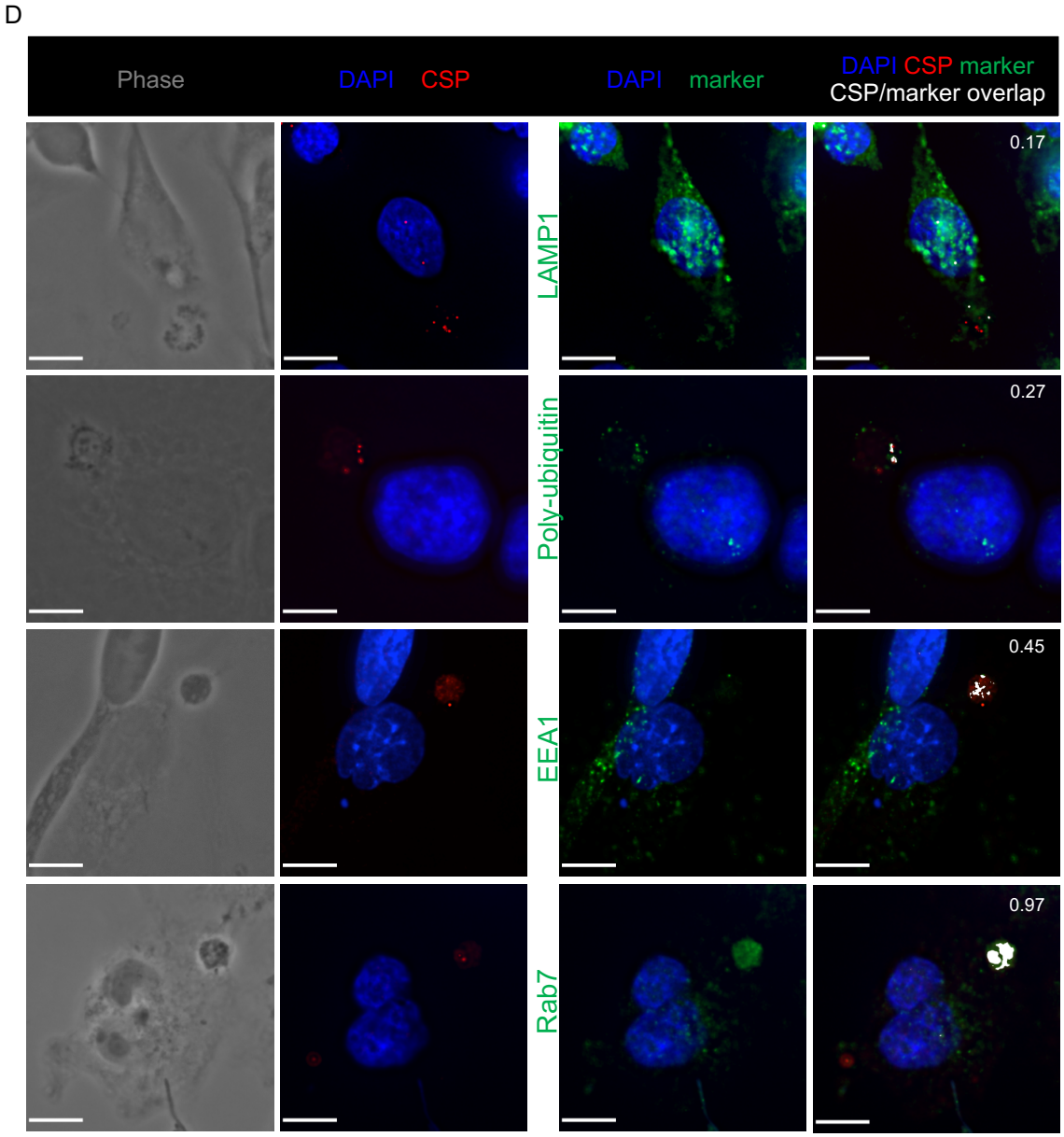

Figure 2-11. Characterization of subcellular compartments retaining CSP in traversed cells.

Co-occurrence of CSP with markers specific for early endosomal (EEA1), late endosomal (Rab7), lysosomal (LAMP1), autophagy-related (LC3and p62) compartments and poly-ubiquitin species was assessed by immunofluorescence microscopy. Representative images are shown for intracellular CSP found in pattern 1 **(A),** 2 **(B)** 3 **(C)** and 4 **(D).** Scale bars = 10 μM. Degree of CSP co-occurrence with each marker (described in Materials and Methods) indicated as percentages of total CSP are shown on each image. Blue = DAPI, red = CSP, green = marker, white = (co-occurrence of CSP and marker)/total CSP.

2.3.11 Proteolytic processing of CSP in traversed cells is impaired by chemical inhibitors of the proteasome, lysosome, and autophagy

Three major cellular proteolytic machineries, the proteasome, lysosome and autophagy, are implicated in the generation of peptide ligands for antigen presentation. Whereas the proteasome and, for some antigens, autophagy-related proteolysis produce CTL epitopes presented on MHC class I, the deviation of antigens to the lysosome likely withdraws them from classical MHC class I presentation pathway (reviewed in Villadangos & Schnorrer 2007). We investigated if specific inhibition of these proteolytic pathways would interfere with the stability and/or intracellular distribution of CSP retained in traversed cells.

Selective chemical inhibition of all three proteolytic pathways, the proteasome (lactacystin), lysosome (chloroquine) or autophagy (spautin-1) from 8-24 and 32-48h pi each resulted in an accumulation of CSP in traversed cells (Figure 2.12A), indicating that all three proteolytic systems regulate the degradation and/or intracellular retention of CSP during the first two days post traversal. On the other hand, only inhibition of the lysosome and autophagosomal pathways, and not the proteasome, led to CSP accumulation from 56 to 72 h pi, suggesting that proteasomes do not play a major role after two days post traversal (Figure 2.12A). At this latest time point, larger aggregates (Patterns 3 and 4) are more predominant than the small puncta observed at 24h pi. This suggests that "CSP bodies" are not susceptible to proteasomal degradation. In contrast, inhibition of either the lysosome or autophagy recapitulated effects seen at earlier time points, indicating that lysosomal and autophagosomal proteolysis are active regulators of

CSP turnover in traversed cells during the first three days following infection (Figure 2.12A).

2.3.12 Distinct cellular proteolytic machineries differentially regulate each CSP-containing compartment

Next, we asked whether the accumulation of CSP following specific inhibition of proteolysis was accompanied by changes in its subcellular distribution. We monitored the frequency of each CSP pattern following specific inhibition of the proteasomal, lysosomal, or autophagosomal processing pathways.

Inhibition of the proteasome at 24 h pi resulted in increased frequencies of cells bearing cytoplasmic diffuse CSP (Pattern 6), probably reflecting the inhibition of otherwise rapid proteasome-dependent turnover of these CSP moieties, like other cytoplasmic antigens (Figure 2.12B). Unexpectedly, inhibition of the lysosome and autophagy also led to an increased frequency of cells containing cytoplasmic CSP. This could suggest that lysosomal-autophagosomal systems degrade cytoplasmic CSP as well. Alternatively, inhibitor-induced perturbance in other vesicular compartments retaining CSP and regulated by these proteolytic systems, results in the release of CSP into the cytosol. In agreement with the latter hypothesis, we observed decreased frequencies of traversed cells with Pattern 1 CSP when treated with lactacystin, chloroquine and spautin-1 (Figure 2.12B).

Surprisingly, at 48 and 72 h pi, chloroquine did not significantly alter frequencies of cells with diffuse cytoplasmic CSP, while inhibition of the proteasome and autophagy decreased the frequency of this cell population (Figure 2.12B). This observation is counterintuitive, as proteasomal inhibition usually results in accumulation of cytosolic

antigens. It is possible that inhibition of the proteasome or autophagy at late time points post traversal sequesters cytoplasmic CSP molecules into other compartments, the integrity or activity of which depend on these proteolytic machineries. This hypothesis is supported by the increased frequencies of cells with heterogeneous punctate staining (Pattern 2) observed in response to proteasomal inhibition at 48 and 72 h pi and accumulation of cells bearing intra- or extracellular "CSP bodies" in cultures treated with the autophagy inhibitor spautin-1 at 48 and 72 h pi (Figure 2.13B).

Overall, the patterns of CSP found in inhibitor treated cells varied significantly from the media control during 8-24h and 56-72h, but not from 32 – 48h pi. (Figure 2.12B) This could suggest that during this time period, CSP was the most stable, or subject to the slowest degradation. The greatest variation in subcellular localization of CSP following inhibitor treatment was seen at 72h pi.

In conclusion, all three major proteolytic systems, lysosome, proteasome and autophagy, simultaneously regulate turnover of CSP in traversed cells. As the latter two are capable of generating MHC class I peptide ligands, we propose that a portion of CSP is available for classical MHC class I presentation in traversed cells throughout the first three days following infection.

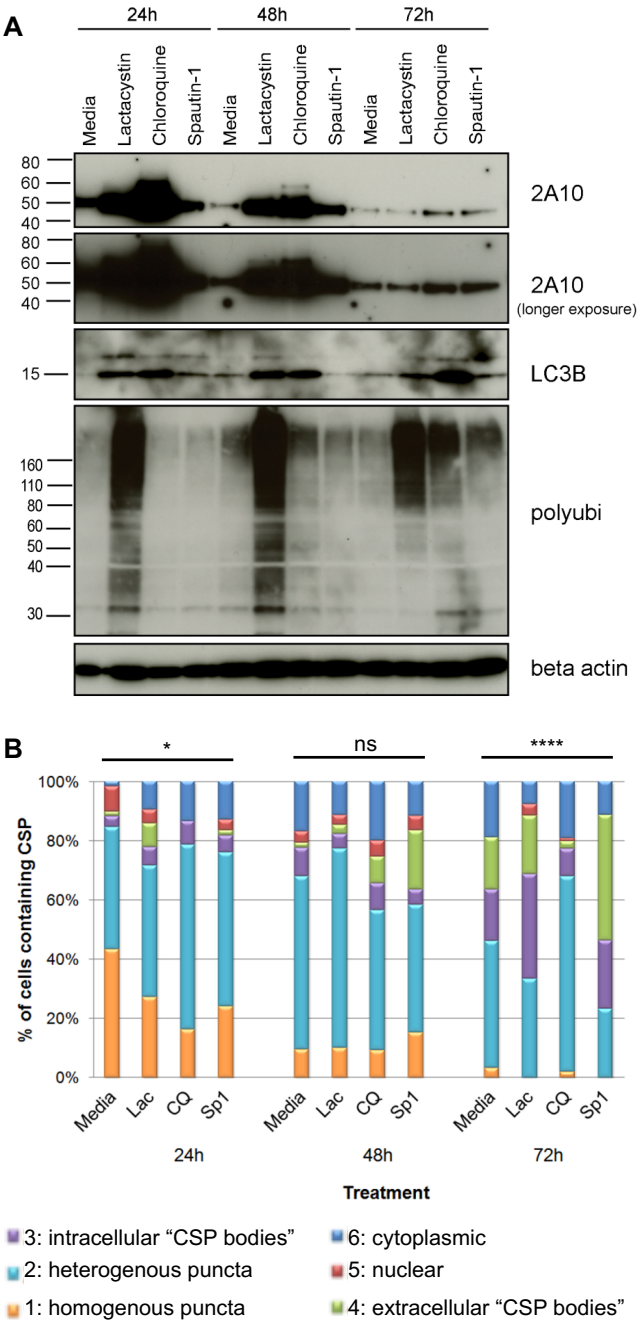

Figure 2-12. Inhibitors of proteasome, lysosome and autophagy differentially regulate processing of CSP residing in distinct subcellular compartments.

(A) Sorted traversed cells were treated with the inhibitors of the proteasome (lactacystin), lysosome (chloroquine) and autophagy (spautin-1) during 16 h prior to 24, 48 and 72 h pi and CSP was detected by western blot. Accumulation of ubiquitin species and detection of 2 forms of LC3B protein were used to confirm the efficacy and specificity of treatments. Actin was used as a sample loading control. **(B)** Distribution of intracellular CSP patterns in response to inhibition of proteasome, lysosome and autophagy; 175 – 200

traversed cells containing CSP were analyzed for each inhibitor treatment in three to six independent experiments. Treatments with inhibitors are described in Materials and Methods. Statistical comparisons were performed between treatments within each time point using Fisher's Exact Test. ns = not significant, * = $p < 0.05$, **** = $p < 0.0001$.

2.4 Discussion

It has previously been described that sporozoites shed CSP apically during gliding motility (Stewart, Michael J., Vanderberg 1988; Stewart & Vanderberg 1991). Hence, we investigated the deposition of CSP into hepatocytes during the second type of sporozoite motility, cell traversal. We documented for the first time that traversed cells retain CSP shed from sporozoites for an extended period of time, up to six days (Figure 2.5). This suggests that CSP from traversed hepatocytes could be available for presentation to T lymphocytes and potentially recruit and/or activate effector CD8+ T cells to the region proximal to infected cells throughout the duration of EEF development.

As CSP is transcribed and protein is present in EEFs for at least five days (Sacci et al. 2006; Mikolajczak et al. 2011), it is theoretically possible that EEFs could remain targets of CSP-specific cytotoxicity throughout this time. While CSP is sequestered into the PV of developing EEFs, studies have shown that it is released by sporozoites prior to PV formation (Hügel et al. 1996) or exported to the hepatocyte cytoplasm (Singh et al. 2007) and that EEFs directly activate CSP-specific CTLs in an HLA-restricted, proteasome dependent manner (Bongfen et al. 2007). Therefore, if CSP could be presented from traversed cells located proximally to invaded cells, we speculate traversed hepatocytes may play a role in controlling infection by recruiting and/or activating CSP-specific CTLs.

To address whether or not CSP in traversed cells has the potential to be presented by MHC class I, we analyzed the subcellular localization and machinery involved in its

turnover. Classical MHC class I presentation requires cytoplasmic or nuclear localization for access to proteasomal processing. Only a minority of CSP in traversed cells (10-20% of CSP-positive cells) visualized by immune fluorescence microscopy was cytoplasmic or nuclear (Figure 2.9), and total CSP amounts detected by immunoblot were only affected by proteasomal inhibition 24 and 48h pi, but not 72h pi (Figure 2.12). This suggests that only a small amount of CSP in traversed cells is available for MHC class I presentation. However, it is possible that the infrequency of cells containing Pattern 6, cytoplasmic CSP, could be due to the fact that the cytoplasm is an effective proteolytic compartment, and these pools of CSP are turned over immediately upon release into the cytoplasm. Furthermore, there is increasing evidence for the presentation of lysosomal/endocytic antigens by MHC class I through non-canonical, TAP-independent pathways utilizing cathepsins (reviewed in Del Val et al. 2011). Thus, we speculate that more traversed hepatocytes than those in which we detected CSP in the cytoplasm or nucleus should have the capacity to present CSP.

In investigating the subcellular localization of CSP deposited in traversed cells, we uncovered a novel phenomenon in the turnover of this parasite antigen: its accumulation in large, stable aggregates. It is common for misfolded and aggregated host proteins to proceed through a sequestration and degradation process, the formation of aggresomes and aggresomes-like induced structures (ALIS), that appears somewhat similar to our observations (Szeto et al. 2006; Johnston et al. 1998). This aggregation could possibly serve both a benefit and detriment to the host. We speculate that Pattern 4 extracellular “CSP bodies,” may persist for an extended period of time in the observed membrane-bound extracellular compartments. If phagocytosed by dendritic cells,

extracellular “CSP bodies” could serve as a long-term reservoir of malaria antigen that is essential for maintenance of memory responses (Cockburn et al. 2010). Alternatively, if not phagocytosed, then this pool of expulsed CSP could serve as a means to evade presentation. In our experimental system, we were unable to investigate these scenarios further.

Altogether, these studies revealed new findings about the deposition and retention of CSP antigen in its natural, post-translationally modified manner by the sporozoite, into the host cell type required for completion of the exo-erythrocytic phase. We found this antigen is retained for an extended period of time with dynamic subcellular localization and is subject to processing by multiple proteolytic systems. These data establish a foundation for investigating the role of traversed hepatocytes as targets in the CSP-specific T cell response.

Chapter 3. INDUCTION OF CD8+ T CELL RESPONSES AGAINST TRAVERSED CELLS: RECOGNITION, ACTIVATION, AND EFFECTOR RESPONSES

Portions of this chapter are adapted from:

Manuscripts:

Trop S#, Arany Z#, Dumoulin P, Ma J, Zhang H, Bream J, Levitsky V and J Levitskaya. Traversal by malaria parasites does not affect the capacity of hepatocytes to trigger CD8+ effector T cell activation.

Trop S, Dumoulin P, Ma J, Zhang H and J Levitskaya. Traversed hepatocytes retain, process and present *Plasmodium falciparum* circumsporozoite protein for CD8+ T cell recognition

#contributed equally

Published work:

Ma J, Trop S, Baer S, Rakhmanaliev E, Arany Z, et al. (2013) Dynamics of the Major Histocompatibility Complex Class I Processing and Presentation Pathway in the Course of Malaria Parasite Development in Human Hepatocytes: Implications for Vaccine Development. PLoS ONE 8 (9): e75321. Doi: 10.1371/journal.pone.0075321.

3.1 Introduction

The liver stage of malaria is an ideal vaccine target because inhibiting infection at liver stage will prevent both clinical malaria and transmission. Furthermore, it is a "practical" target as 1) low parasite numbers in the liver represent a "bottleneck" of *Plasmodium* infection and 2) the immune system is able to abrogate infection through major histocompatibility complex (MHC) class I-restricted CD8+ cytotoxic T cell (CTL) recognition of infected hepatocytes (reviewed in (Van Braeckel-Budimir & Harty 2014; Tse et al. 2011)).

Plasmodium EEFs develop within the PV, a controlled environment sequestered from the host cytosol in which the parasite can avoid innate immune signals and proteolytic machinery of the host. The double plasma membrane of the PV is thought to strictly regulate trafficking between the parasite compartment and host cytosol, allowing only for import and export of molecules beneficial for parasite growth (reviewed in (Ingmundson et al. 2014)). Parasite proteins containing *Plasmodium* export elements (PEXEL) RxLxE, including CSP, liver-stage antigen 3 (LSA-3), IBIS1 and PFB0105c are believed to be exported outside the PV (Marti et al. 2004; Singh et al. 2007; Ingmundson et al. 2012; Siau et al. 2008). While there is evidence that several liver stage antigens may be exported to the cytosol and, therefore, may become targets for CTL responses (Plebanski, Aidoo, et al. 1997; Aidoo et al. 2000; Bongfen et al. 2007; Toure-Balde et al. 2009; Sedegah et al. 2013), this process is still under scrutiny (Cockburn et al. 2011). Nonetheless, the requirement for MHC class I-restricted CTL responses against

liver stage antigens is well-documented, as is the case with other intravacuolar bacterial and protozoan infections (reviewed in (Blanchard & Shastri 2010).

As discussed in Chapter 2, CSP, an important target of protective CD8+ T cell responses following vaccination, was retained in hepatocytes traversed by *P. falciparum* sporozoites. Furthermore, CSP detected in traversed cells was long-lived, retained in several, dynamic subcellular compartments, and was subject to degradation by multiple cellular proteolytic machineries, including the proteasome, which is important for MHC class I presentation. Importantly, traversed cells remained viable for several days.

It was still not clear, however, whether breaching of the hepatocyte plasma membrane and release of the cellular content into the extracellular milieu (Carrolo et al. 2003; Leirião et al. 2005) affects the ability of traversed hepatocytes to effectively stimulate MHC class I-restricted CTL responses. In particular, it is not known whether or not traversed hepatocytes can sufficiently activate CD8+ T cells to execute the full array of T cell effector functions, including cytokine secretion, cytotoxic granule release, and cell division. It is also unknown if the process of traversal affects the immunogenicity of neighboring, nontraversed hepatocytes.

We speculated that this CSP retained in traversed cells might serve as another source, in addition to infected hepatocytes, of presented peptides to induce CTL responses. CSP induces both humoral and cellular immune responses in naturally infected and vaccinated hosts, including the well-studied CD8+ CTL-mediated control of malaria liver stages (reviewed in Hafalla et al. 2011). Therefore, identification of antigen presenting cells capable of presenting liver stage antigens on MHC class I to CD8+ T

cells remains an important goal. Thus, we sought to determine if traversed cells could present CSP to trigger effector functions of CTLs.

Here, we utilized two experimental models to 1) assess T cell activation as a measure of the overall stimulatory capacity of the hepatocyte while bypassing endogenous antigen presentation and 2) specifically determine the ability of traversed hepatocytes to endogenously process and present a CTL epitope derived from the C-terminus of CSP. We performed an extensive comparative analysis of the capacity of traversed, non-traversed and non-infected hepatocytes to serve as stimulators of MHC-class I-restricted CTL responses. Our data demonstrate that traversed, non-traversed and non-infected hepatocytes had similar abilities to trigger the full array of T cell activation events, suggesting that effective parasite-specific CTL responses may take place in close proximity to hepatocytes harboring developing EEFs. Additionally, traversed cells specifically induced IFNγ cytokine production by CSP-specific human CTLs. Activation of these CTLs by infected cultures of HC-04 cells limited the development of *P. falciparum* EEFs.

3.2 Materials and Methods

Surface staining and flow cytometry of HC-04 cells

Traversed and nontraversed HC-04 cells were incubated with antibodies against HLA-ABC, CD54 (BD, Franklin Lakes, NJ), HLA-E (eBioscience, San Diego, CA) or relevant isotype control antibodies directly conjugated to fluorophores. Data were acquired on FACSCalibur^{TM} (BD) using CellQuest Pro software (BD).

CSP-coding DNA plasmids

Expression vectors coding for two forms of CSP, CSPfull and N-terminal cleavage product CSPshort were generated by Elian Rakhmanaliev and Jinxia Ma (described in detail in (Ma et al. 2013)). Plasmids were utilized by for transient ectopic expression of CSP described below.

The pVITRO2-neo-mcs (InvivoGen, CA, USA) contains two separate multiple cloning sites and allows the ubiquitous and constitutive co-expression of two genes of interest. Sequence encoding mouse CD8α was PCR amplified and inserted into one of the cloning sites. Surface expression of mouse CD8α serves as a heterologous surface marker of transfection efficiency in human cells. Sequences coding for either "full" or "short" length CSP from the *P. falciparum* 3D7 strain were cloned into the second cloning site. The *P. falciparum* CSPshort protein corresponds to the mature form of CSP produced by proteolytic cleavage of full length CSP during parasite interaction with the host cell plasma membrane (Coppi et al. 2005).

Transient transfection with plasmids encoding *P. falciparum* CSP

Adapted from (Ma et al. 2013).

HC-04 cells were transfected with pVITRO2-CD8, pVITRO2-CD8/CSPfl, pVITRO2-CD8/CSPsh plasmids using Lipofectamine LTX (Life Technologies, Grand Island, NY). Expression of CSP was detected either by western blot in transfected cells at 24 h post transfection or by immunofluorescence using CSP-specific mouse monoclonal antibody 2A10 (kind gift of Dr. F. Zavala, JHMRI, JHU). Beta actin-specific antibody AC15 (Sigma-Aldrich, St. Louis, MO) was used to verify equal loading of cell lysates.

Blots were developed using anti-mouse IgG-HRP (GE Healthcare, Pittsburgh, PA) and Super Signal West Dura Extended Duration Substrate (Thermo Scientific, Waltham, MA).

Twenty four and 48 h post-transfection, surface expression of mouse CD8α was assessed by staining with anti-mouse CD8α-RPE or IgG2b-RPE isotype control antibody (Invitrogen, Grand Island, NY) and MHC class I expression was assessed in CD8α+ cell populations as described above. Data acquisition was performed using FACSCaliburTM (BD Biosciences, San Jose, CA). To monitor MHC class I pathway and its responsiveness to pro-inflammatory cytokines in CSP transfected cells, hepatocytes were sorted by MoFlo Cell Sorter (Beckman Coulter, Indianapolis, IN) 24 h post-transfection, treated with AS or left untreated (as described above) for additional 24 h and real-time PCR analysis was performed for HLA-A heavy chain, β2m and TAP1 genes at 48 h post-transfection. In parallel, class I heavy chain expression was assessed by western blot using specific rabbit polyclonal antibody (kind gift of Dr. H. Ploegh).

Analysis of CTL activation by flow cytometry

A CD8+ HLA-A*0201 -restricted human cytotoxic T lymphocyte (CTL) clone LF8, specific for EBV BMFL1 (280-288) CTL epitope GLCTLVAML (GLC) was generated as previously described (Levitsky et al. 1998) and used to assess T cell activation induced by HLA-A*0201-positive HC-04 cells pre-pulsed with the synthetic GLC peptide. HLA-A*0201 -specific allogeneic CD8+ CTL clone was used to evaluate its activation by HC-04 cells and primary HLA A2-positive human hepatocytes.

BK-B5 CD8+ human CTL clone specific for an EBV EBNA4 (416-424) epitope IVTDFSVIK (IVT) (Levitsky et al. 1998) was used to evaluate the bystander effect of LF8 clone activation on traversed HC-04 cells. Hepatocytes were separated into traversed

and nontraversed populations at 2 h pi and pre-incubated with GLC peptide at concentration of 10^{-6} M or 10^{-8} M for 1 h followed by unbound peptide removal by washing. Peptide-pulsed hepatocytes were co-cultured with CTLs at 3:1 CTL-to-hepatocyte ratio for 4 h in complete medium containing Golgi Plug (BD Biosciences, San Jose, CA) followed by the concomitant surface staining with CD8-specific mouse monoclonal antibody conjugated to Pacific Blue and one of the following mouse monoclonal antibodies: anti-CD25, anti-CD69 or anti-PD-L1, all conjugated to phycoerythrin (PE). Cells were washed, fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences, San Jose, CA) and incubated with one of the following antibodies: anti-IL-2, anti-IFNγ or anti-TNFα, all conjugated to allophycocyanin (APC). Relevant IgG1 isotype control antibodies, conjugated to PE or APC were used. All antibodies were obtained from BD Biosciences (San Jose, CA). Samples were acquired with a LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Multiplex cytokine detection

For cytokine detection in the supernatants of activated T cells, CTL clone LF8 was stimulated with HC-04 cells either not exposed to sporozoites ("uninfected"), or sorted *Pf* traversed or nontraversed cells, pulsed with exogenous cognate peptide as described above. CTLs (10^5) were stimulated with hepatocytes (1:1 ratio) at 30 h pi and supernatants were collected after 16 h of co-incubation, aliquoted and stored at -80C prior to analysis using Meso Scale Discovery Proinflammatory Panel 1 V-PLEX kit (Rockville, MD) and on Meso Scale Discovery SECTOR Imager 2400 following manufacturer's protocols (Johns Hopkins School of Public Health Flow Cytometry Core Facility).

T cell degranulation assays

HC-04 cells, either uninfected or separated into traversed and nontraversed populations 2 h pi, were pre-pulsed with GLC peptide at 24 h pi and co-incubated with LF8 CTLs at 1:3 T cell-to-hepatocyte ratio for 4 h in the presence of anti-CD107-PeCy5 antibody or IgG1-PeCy5 isotype control antibody. Cells not pulsed with peptide were used as stimulators to determine the basal levels of exocytosis in CTLs. Samples were acquired with a LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

T cell proliferation assays

LF8 CTLs were labeled with carboxyfluorescein diacetate succinimidyl ester, CFSE, (Life Technologies, Grand Island, NY) according to the manufacturer's protocol and incubated with noninfected, nontraversed or *Pf* traversed HC-04 cells pre-pulsed with GLC peptide at 10^{-6} M or 10^{-8} M concentration. CFSE fluorescence was measured by flow cytometry in the CD8+ cell population at day 5 post triggering. CFSE fluorescence intensity of nonstimulated cells was used as a control for basal proliferation of T cells. Stimulations were performed in triplicate cultures.

Analysis of endogenous CSP presentation by traversed cells

Human CTLs specific for HLA-A*0201 restricted epitope *Pf*$CSP_{327-335}$ (YLNKIQNSL) previously described (Bonelo et al. 2000) were generously provided by G. Corradin. Custom Class I MHC tetramer for this epitope was kindly provided by the NIH Tetramer Core Facility (Emory University, Atlanta, GA). CTLs were stimulated by HC-04 cells either sorted (into traversed and nontraversed populations) from cultures infected with *P. falciparum* sporozoites, or unsorted infected and noninfected cultures.

HC-04 cells pulsed with exogenous *Pf*CSP$_{327-335}$ peptide (10^{-6} M) were used as a positive control. CTL activation was measured by the Miltenyi IFNγ Secretion Assay (Miltenyi Biotec Inc., San Diego, CA) following a 3 h stimulation according to manufacturer's protocols.

3.3 Results

3.3.1 Immune surface phenotype is unaltered in traversed hepatocytes

Pathogens alter MHC class I antigen presentation via multiple mechanisms leading to immune escape from CTL-mediated surveillance by downregulation of MHC class I complexes or induction of inhibitory HLA-E and HLA-G molecules (Abele and Tampe, 2011; Amiot et al., 2014; Basta and Bennink, 2003; Sullivan et al., 2008). We found that total levels of assembled MHC class I complexes on sorted, traversed hepatocytes, as measured by staining with HLA-A, B, C-specific antibodies, were similar to that on nontraversed cells at during at least the first 72 h pi with *P. falciparum* sporozoites (Figure 3.1). While marginal constitutive expression of HLA-E was observed in HC-04 cells, it was not altered by sporozoite traversal. Interestingly, ICAM-1, an essential component of the immunological synapse on antigen presenting cells (reviewed in (Thauland and Parker, 2010)) was more highly expressed on traversed hepatocytes, at least up to 72 h pi (Figure 3.1). The same trends were observed in HC-04 cells traversed by *P. berghei* (data not shown). HLA-G and HLA-DR,DP,DQ expression were not detected in neither noninfected nor infected cell cultures (data not shown). In summary, the cell surface phenotype of hepatocytes traversed with *P. falciparum* sporozoites is compatible with the ability of these cells to present antigens on MHC class I.

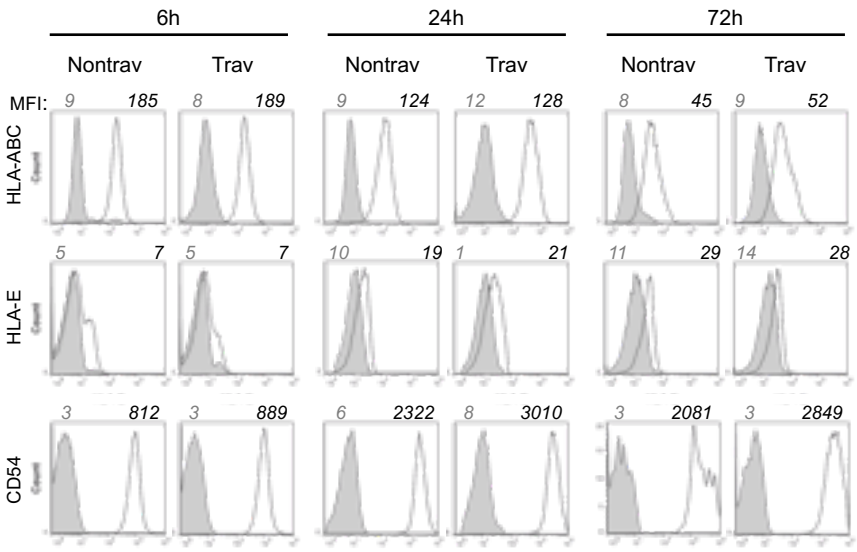

Figure 3-1. Traversed hepatocytes maintain expression of MHC class I and ICAM-1.

Surface expression of total HLA class I, HLA-E and ICAM-1 was detected by immunostaining and flow cytometry on sorted traversed and nontraversed HC-04 at indicated times pi, as described in Materials and Methods. One representative experiment is shown. Numbers indicate geoMFI of cells stained with isotype control antibody (shaded histograms) or indicated specific antibody (open histograms). Data are representative of three independent experiments.

3.3.2 Transient ectopic expression of CSP does not interfere with steady-state or cytokine-induced expression of MHC class I molecules

Data generated by S. Trop, published in (Ma et al. 2013)

Upregulation of MHC class I and II expression by APCs in response to pro-inflammatory cytokines is critical in orchestrating efficient immune responses against infections. Many pathogens subvert host immune surveillance by interfering with cytokine signaling (reviewed in (Alto & Orth 2012)). CSP, which is found in traversed cells (Chapter 2), has been shown to inhibit TNFα-induced translocation of NF-κB into the nucleus of HepG2 or HeLa cells, thereby preventing transcription of NF-κB responsive genes (Singh et al. 2007). These results prompted us to investigate whether

CSP expression alone could be sufficient to inhibit the inducible expression of genes controlling antigen processing and presentation. While we did not observe changes in surface expression of MHC class I in traversed cells retaining CSP deposited by sporozoites (Figure 3.1), we investigated if the ectopic expression of CSP, which yields different subcellular localization and quantities of protein, may have an effect on cytokine responsiveness.

To examine the effect of CSP on MHC class I expression, we ectopically expressed the two major forms of *P. falciparum* CSP: the full length protein (CSPfl) and its truncated, "mature" form (CSPsh) that results from proteolytic cleavage during sporozoite interaction with host hepatocyte plasma membrane (Coppi et al. 2005) (Figure 3.2A, B). CSP expression was driven from a bicistronic plasmid vector also encoding surface protein mouse CD8α (mCD8α), which served as a marker for transfection, allowing flow-cytometric detection and sorting of live CSP+ cells. HC-04 cells expressing mCD8α alone (vector control) or co-expressing with CSPfl or CSPsh were isolated 24 h after transfection or directly detected using immunostaining and flow cytometry (Figure 3.2).

In the course of natural immune responses, hepatocytes are likely to be exposed to a plethora of cytokines produced by activated T cells. Therefore, we assessed potential modulation of MHC class I machinery in HC-04 transfected with either mCD8α vector control, CSPfl, or CSPsh plasmids after treatment with recombinant TNFα and IFNγ or with supernatant collected from activated human CTLs ("activated supernatant"). Neither CSPfl nor CSPsh expression affected the steady state nor cytokine-induced mRNA levels of MHC class I heavy chain, β_2M, or TAP1 24 h post-transfection (Figure 3.2C). In line

with this observation, surface expression of MHC class I was also unaltered by CSP expression under the same treatment conditions (Figure 3.2D, E). Likewise, expression levels of MHC class I heavy chain assessed by immunoblotting of total cell lysates were comparable between sorted CSP- and vector-transfected HC-04 cells (Figure 3.2F). Collectively, these results indicate that plasmid-driven expression of CSP does not interfere with the antigen presenting machinery in HC-04 human hepatocytes, contrary to the reports of Singh and colleagues (Singh et al. 2007). In conclusion, using two independent experimental models: (a) traversed cells retaining CSP deposited by sporozoites and (b) cells ectopically overexpressing CSP outside of the context of *Plasmodium* infection, we consistently demonstrated that CSP-containing hepatocytes remained competent to present antigen via MHC class I and activate CD8+ T cells in antigen-dependent manner.

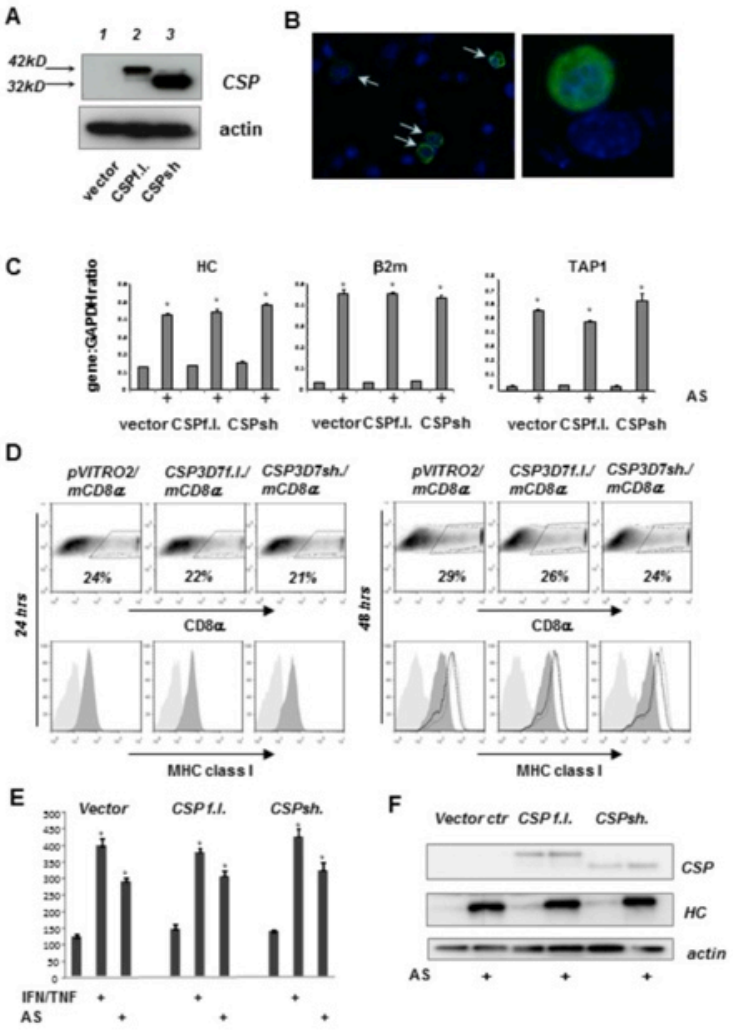

Figure 3-2. CSP does not alter steady-state of induction of MHC class I

(A) Expression of the full length (CSPf.l.) or mature "short" (CSPsh) form of *P. falciparum* CSP was detected by western blot 24 h post-transfection of HC-04 cells. **(B)** Cellular distribution of full length CSP 24 h post-transfection was visualized with a CSP-specific antibody (green) and immunofluorescence microscopy. Nuclei were stained with DAPI (blue). Arrows indicate transfected cells. **(C)** Real-time PCR analysis of MHC class I heavy chain, β2-microglobulin and TAP1 gene expression in cells transiently expressing *P. falciparum* CSP. HC-04 cells transfected with the control vector plasmid or plasmid encoding either full length or short CSP were treated with AS (10% v/v) 4 h post-transfection or left untreated. Transfected cells were isolated 24 h later based on surface expression of mouse CD8α. Mean ± SD of the assay triplicates. All p* <0.0002 and indicate differences between control and AS-treated samples. **(D)** Percentages of cells transiently expressing CSP were identified by CD8α co-expression (upper panels) and MHC class I was assessed by flow cytometry (lower panel, light gray histograms - isotype control, dark gray histogram - MHC class I specific antibody). A proportion of cells exposed to either AS (10% v/v) or a mixture of recombinant TNFα and IFNγ at 24 h post-transfection was further assessed for MHC class I expression at 48 h (light gray histograms - isotype control antibody, dark gray histogram - MHC class I in untreated cultures, solid line histogram – cultures treated with recombinant cytokines, dotted line

histogram – cultures treated with AS). Data from one representative experiment. **(E)** The means ± SD of MFI for specific MHC class I staining obtained in 3 independent experiments. All p* <0.0001 and indicated differences between control and treated samples. **(F)** Expression of MHC class I heavy chain (HC) in total cell lysates of HC-04 cells transfected with CSP-expressing plasmids was assessed by western blot. Treatment with AS was done as described for D.

3.3.3 Direct stimulation with traversed cells triggers activation of CD8+ T cells similarly to nontraversed cells

It is generally accepted that the full program of CD8+T cell activation includes cytokine secretion, rearrangement of cell surface antigens at the immunological synapse, release of cytotoxic granules, and cell proliferation (reviewed in (Suni et al. 2005)). Next, we comprehensively assessed the ability of traversed cells to trigger activation of CTLs.

Traversed cells induce cytokine secretion from specific CD8+ T cells

First, we analyzed cytokine production in effector CTLs by two methods: intracellular staining, to measure numbers of responding cells, and multiplex cytokine detection in supernatants, revealing the total amount of multiple cytokines secreted from an entire culture of effector T cells.

Upon activation of GLC-specific CTL line LF8 with peptide-pulsed nontraversed or traversed HC-04 cells, no differences in the number of cells producing IFNγ, TNFα or IL-2 or MFI were detected by intracellular staining and flow cytometry, neither at 6 nor at 24 h pi (Figure 3.3A, B, C). Furthermore, sporozoite-exposed HC-04 populations (separated into nontraversed and traversed) did not differ in their stimulatory capacity from cells not exposed to sporozoites (designated as “non-infected”). These findings were reproducible with an allogeneic HLA-A*02-specific CTL clone as a responder (Figure 3.4A), as well as with HC-04 cells traversed with *P. berghei* sporozoites (data not shown). Taken together, these data indicate that traversed hepatocytes can stimulate cytokine

secretion from CTLs as efficiently as nontraversed or noninfected hepatocytes, independently of CTL or hepatocyte donor.

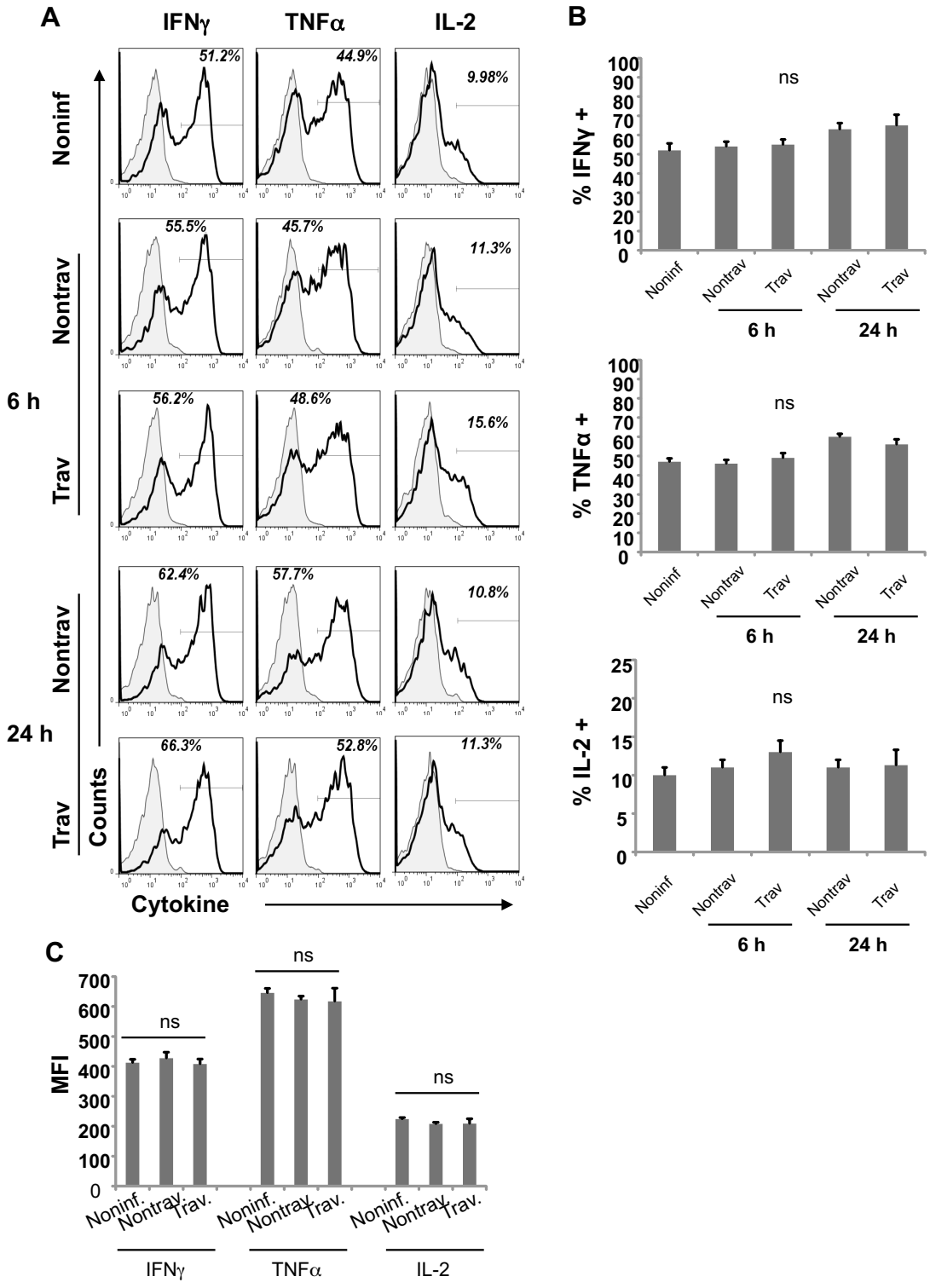

Figure 3-3. Activation of peptide-specific CTLs by traversed and nontraversed hepatocytes pulsed with GLC peptide

Cell sorting was done 2 h pi and hepatocytes were used as stimulators at 6 and 24 h pi. Intracellular IFNγ, TNFα and IL-2 cytokine detection in GLC peptide-specific CTL clone

LF8 was performed after 4h of co-culture, as described in Materials and Methods. **(A)** Representative histograms are shown. Shaded histograms define isotype control staining intensity and outline gating strategy. Numbers are percentages of cells with high cytokine-specific fluorescence. **(B)** Summary of data obtained from three independent cultures analyzed for each time point and the cytokine. ns, no significant differences were observed comparing all groups by ANOVA **(C)** MFI, mean fluorescence intensity for one representative experiment of 3 performed is shown. ns, no statistical differences were observed comparing groups within each cytokine by ANOVA.

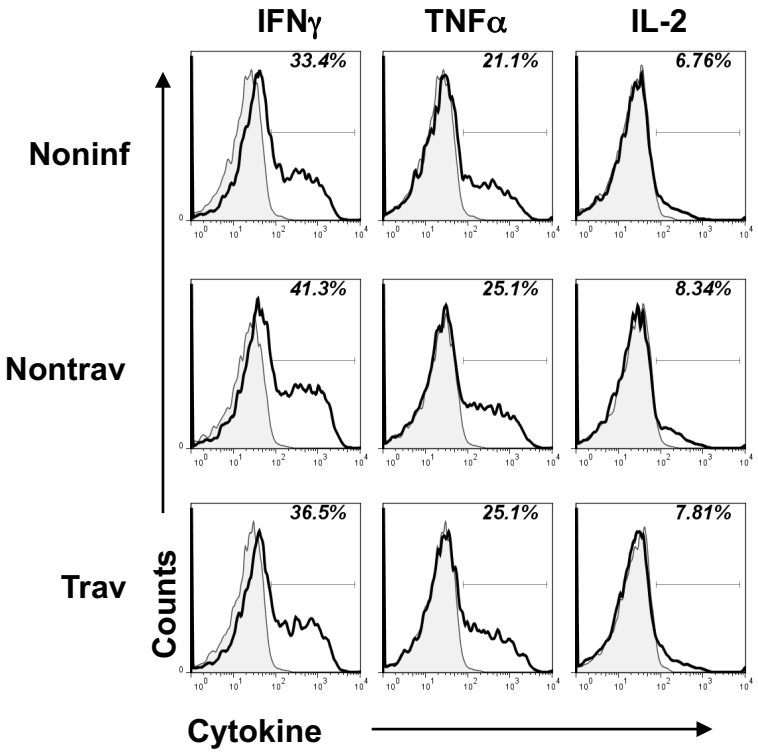

Figure 3-4. Intracellular cytokine detection in HLA-A*02-specific allogeneic T cells

Allogeneic HLA-A*02-specific T cells were activated on traversed and nontraversed HC-04 cells 24 h post infection. Gating strategy and controls are defined as described in the legend to Figure 3.3. Representative histograms are shown for each cytokine.

Next, using multiplex cytokine detection methods to measure overall quantities of cytokines released into cell culture supernatant by T cells upon activation on their respective targets, we observed no defects in cytokine production by CTLs activated by traversed, as compared to nontraversed HC-04. In fact, there was an increase in production of IFNγ, IL-13, IL-2, IL-4, IL-8 and TNFα by CTLs activated on traversed

cells (Table 3.1). Interestingly, exposure to nontraversed cells also resulted in more efficient release of the same cytokines by CTLs, as compared to stimulation with noninfected hepatocytes.

Table 3-1. Cytokines (ng/mL) released by CTLs activated on HC-04 cells 30 h pi

Stimulator	**GLC pep**	IFNγ	IL-10	IL-12 p70	IL-13	IL-1β	IL-2	IL-4	IL-6	IL-8	TNFα
Noninfected	-	48.8	0.1	0.2	17.2	0.0	34.8	0.2	0.2	12.3	0.2
Noninfected	10^{-8} M	101.1	0.3	0.0	32.5	0.1	42.3	0.6	0.4	50.6	3.2
Noninfected	10^{-7} M	1140.5	0.4	0.6	244.4	0.1	223.8	27.8	0.7	199.7	103.9
Nontraversed	-	55.1	0.1	0.2	17.7	0.1	32.5	0.1	0.3	12.2	0.5
Nontraversed	10^{-8} M	95.1	0.1	0.3	31.3	0.1	36.1	0.5	0.5	32.4	2.9
Nontraversed	10^{-7} M	2590.9	0.9	0.6	416.6	0.1	462.6	51.0	0.6	224.9	235.9
Pf traversed	-	50.8	0.1	0.2	16.2	0.2	31.1	0.2	0.2	17.1	0.5
Pf traversed	10^{-8} M	140.3	0.4	0.5	52.6	0.1	49.9	1.6	0.6	100.2	6.1
Pf traversed	10^{-7} M	4867.8	1.1	0.9	473.8	0.2	771.7	83.9	0.8	355.2	451.2

Traversed hepatocytes induce activation markers on the surface of responding CTLs

In parallel with cytokine release, activated CD8+ T cells alter expression of a number of cell surface antigens involved in signaling events. Upregulation of CD69, a signaling membrane glycoprotein involved in induction of T cell proliferation, CD25, the IL-2R α subunit, and programmed death ligand 1 (PD-L1) were induced on resting CD8+ effector T cells to a comparable extent after incubation with noninfected, nontraversed or traversed HC-04 cells at 24 h and then declined in all three cultures at 48 h of incubation (Figure 3.5).

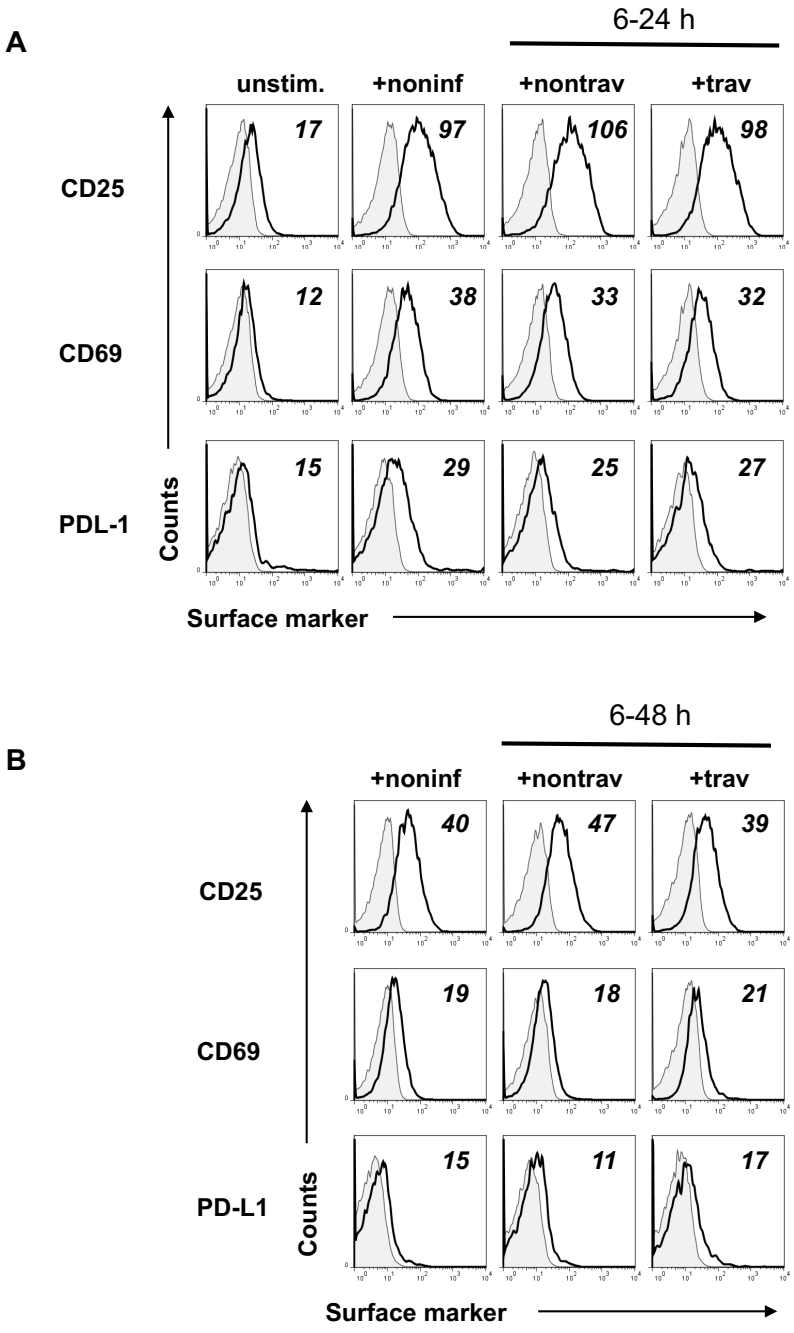

Figure 3-5. Cell surface activation markers expressed by CTLs specifically triggered by traversed or nontraversed hepatocytes.

Cell sorting was done 4 h pi and peptide-pulsed hepatocytes were used as stimulators for GLC peptide-specific CTL clone LF8 at 6 h pi. Expression of CD25, CD69 and PD-L1 was assessed by immunostaining followed by flow cytometry at 24 h **(A)** and 48 h **(B)** post incubation. Unstimulated T cells were used to define steady levels of CD25, CD69 and PD-L1 expression. Data from one representative of three experiments performed for each marker. Shaded histograms - isotype control antibodies, numbers indicate MFI of marker-specific antibody staining.

CD8+ T cells efficiently degranulate in response to stimulation by hepatocytes traversed with *P. falciparum*

Degranulation is believed to be the major effector function of CD8+ T cells resulting in perforin-granzyme mediated cell death of the target (reviewed in (Ewen et al. 2012)). CD107, a component of the lysosome membrane, is often used as a marker of cytotoxic granule release by NK and T cells (Betts et al. 2003). Six to eight percent of T cells had low surface expression of CD107 prior to specific stimulation, whereas in CTL cultures incubated with noninfected, nontraversed or traversed hepatocytes pre-pulsed with the relevant CTL peptide epitope, the number of CD107-positive cells increased by several fold, indicating efficient degranulation (Figure 3.6). All three hepatocyte populations induced T cell degranulation similarly.

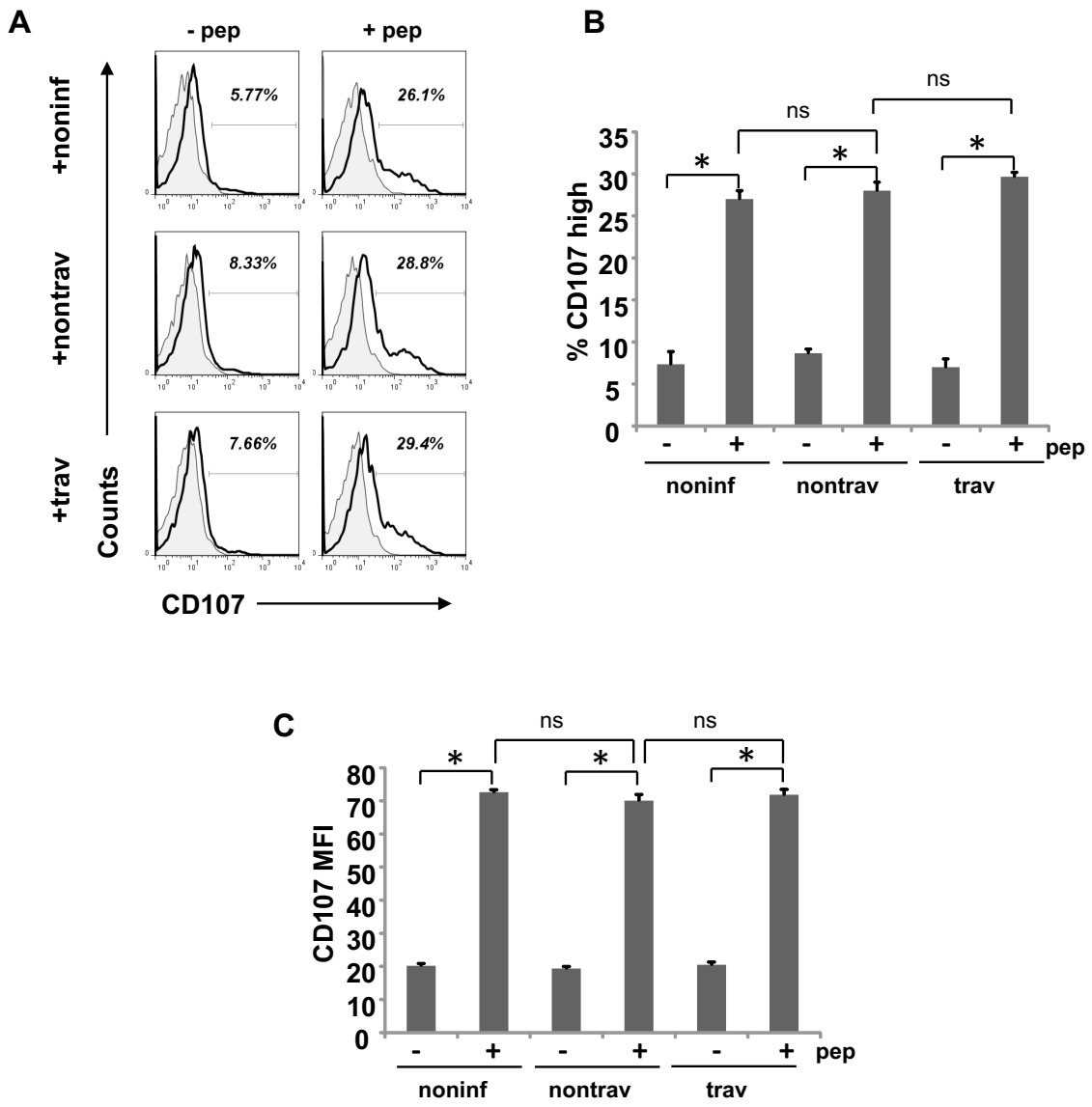

Figure 3-6. Degranulation of CTLs induced by traversed and nontraversed hepatocytes.

Expression of CD107 degranulation marker was measured in CTLs incubated with traversed, nontraversed or noninfected HC-04 cells pre-pulsed with GLC peptide as described in Materials and Methods. Cells without peptide served as control stimulators. **(A)** Representative histogram plots are shown. Shaded histograms (isotype controls) demonstrate gating strategy. Numbers reflect percentages of cells with high CD107-specific fluorescence. **(B)** Summary of data obtained from three independent cultures. **(C)** CD107-MFI for one representative of two independent experiments is shown. ns, non significant by T tests with multiple comparison corrections, * $p<0.001$

Hepatocytes traversed by *P. falciparum* sporozoites trigger specific proliferation of CD8+ CTLs

Amplification of the antigen-specific T cell pool is a desirable outcome of specific T cell receptor (TCR) triggering. CTL proliferation was measured as a decrease in fluorescence of CFSE-labeled T cells following stimulation with noninfected, nontraversed or traversed hepatocytes pre-pulsed with the relevant peptide at two different concentrations (Figure 3.7A). T cell proliferation, measured as percentages of $CFSE^{low}$ CD8+ T cells co-incubated with the three different hepatocyte populations, was dependent on the specific peptide concentration and was not affected by hepatocyte traversal (Figure 3.7B). Accordingly, no significant differences were observed in the mean fluorescent intensities of CFSE that reflect the average number of divisions of each T cell (Figure 3.7C). Thus, traversal does not affect the ability of hepatocytes to trigger specific proliferation of CD8+ CTLs.

Altogether, these data show that traversed hepatocytes have no intrinsic alterations in the capacity to stimulate CTL effector functions as compared to nontraversed or noninfected cells. When providing specific exogenous HLA-A*02-restricted peptide on the surface of target hepatocytes, all three populations of HC-04 cells stimulated secretion of pro-inflammatory cytokines, upregulated expression of surface markers of activation, triggered release of cytotoxic granules and proliferation.

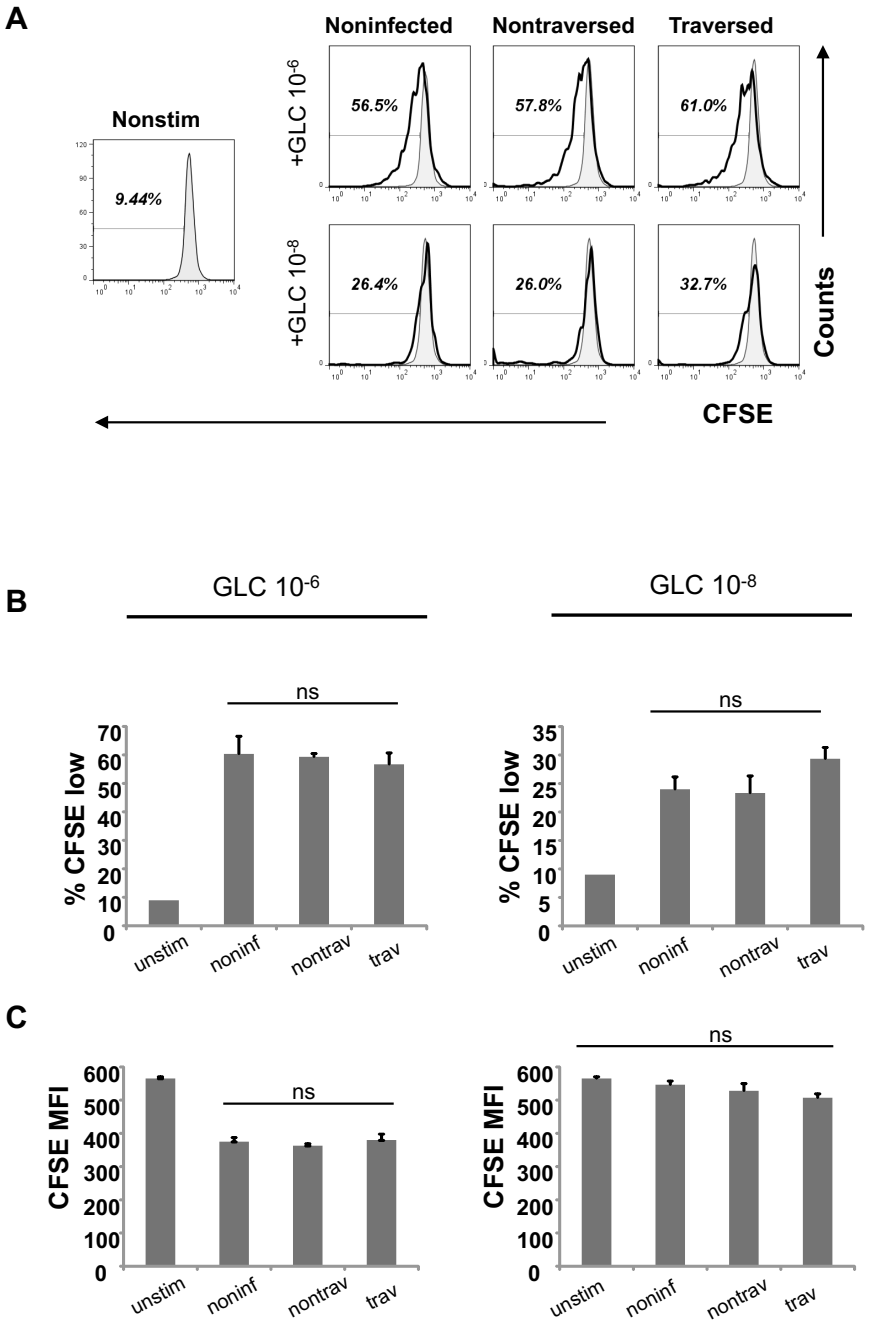

Figure 3-7. Proliferation of CTLs triggered by traversed and nontraversed hepatocytes.

Proliferative capacity of LF8 CTLs in response to noninfected, nontraversed or traversed hepatocytes pre-pulsed with the GLC peptide at the indicated concentrations was measured as CFSE dilution at day 5 post triggering. CFSE fluorescence intensity of nonstimulated cells was used as a control to establish a threshold for "$CFSE^{low}$" cell population arbitrary defined below 10%. **(A)** Representative histograms are shown for each stimulator. Numbers are percentages of "CFSE low" cells. **(B)** Summary of data obtained from three independent cultures analyzed for each stimulator. Percentages of $CFSE^{low}$ T cells and mean fluorescence intensities of CFSE are shown for each stimulation condition. ns, not significant comparing groups under line by ANOVA.

3.3.4 Soluble factors released either by traversed hepatocytes or by T cells activated on traversed hepatocytes do not impose in-*trans* effects on T-cell activation

Cytoplasmic contents of traversed cells are released into culture medium during sporozoite traversal *in vitro* (Coppi et al. 2007). Therefore, it is conceivable that cellular contents released, as well as soluble factors produced by activated CTLs at the site of immune activation, may affect nearby cells, including both target hepatocytes and effector T cells. To address possible *trans* effects of traversal on neighboring cells, we activated CTLs with specific peptides in the presence of cell culture supernatants collected from sorted traversed and nontraversed HC-04 cells every 12 hours during the first 4 days pi. No differences in the efficacy of T cell activation were observed under these conditions (Figure 3.8), indicating that traversal of hepatocytes is unlikely to influence local T cell activation in *trans*.

To determine whether T cell activation by traversed cells can influence cell surface phenotype in a bystander manner through production of soluble factors, we monitored surface expression of PDL-1 and HLA-A*02 on hepatocytes, both of which are known to be upregulated in response to pro-inflammatory cytokines. We found that hepatocytes exposed to soluble factors released by CTLs upon activation by noninfected, nontraversed, or traversed cells had comparable upregulation of PDL-1 and HLA-A*02 (Figure 3.8B), demonstrating similar in *trans* effects mediated by T cells activated on traversed and nontraversed cells.

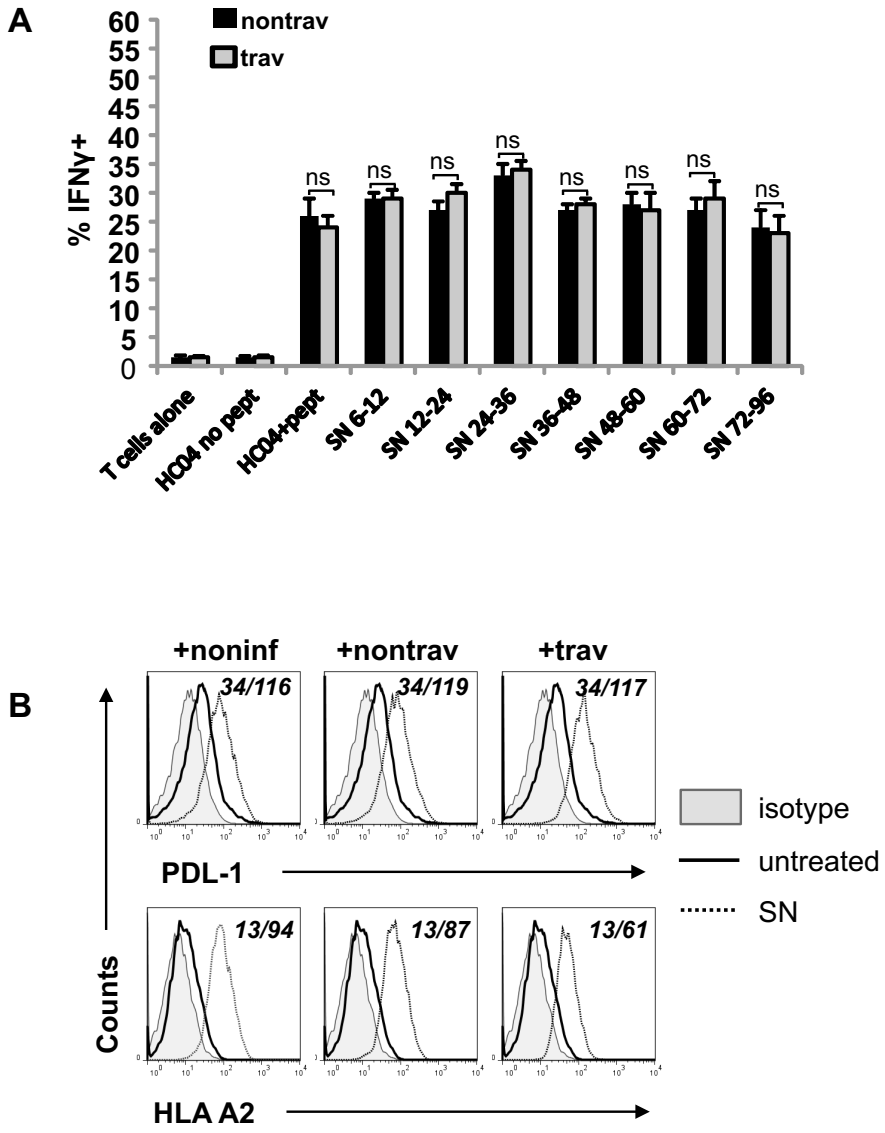

Figure 3-8. Traversal of hepatocytes does not mediate in-trans effects modulating T cell activation.

(A) Intracellular IFNγ detection in CTLs activated in the presence of supernatants obtained from traversed or nontraversed hepatocyte cultures and collected at different time points (6-96h) post infection. Each bar is a summary of data obtained from three independent CTL cultures. **(B)** HLA-A*02 and PD-L1 expression on HC-04 cells incubated with soluble factors released by CTLs activated on traversed and nontraversed cells. GLC-specific CTLs were activated on targets 24 h pi and culture supernatants collected 16 h post-activation were applied onto HC-04 cells for another 24 h. Expression of HLA-A*02 and PD-L1 was assessed by immunostaining followed by flow cytometry. Shaded histograms define isotype control staining intensity and outline gating strategy. Numbers indicate MFI of marker-specific staining observed in control and SN-treated hepatocyte cultures.

3.3.5 Traversed cells support endogenous processing and presentation of retained CSP and activate parasite-specific CD8+ T cells

After establishing the ability of traversed hepatocytes to serve as non-professional APCs, we next determined if parasite-specific antigens could be presented by traversed hepatocytes. The possibility of presentation of parasite antigens from invaded and traversed hepatocytes remains an important question in the understanding of CTL responses to liver stage malaria infection (Bongfen et al. 2007). Using human CTL clone specific for HLA-A*02- restricted epitope *Pf*$CSP_{327-335}$ (YLNKIQNSL)(Bonelo et al. 2000), we assessed the ability of *Pf* -traversed HC-04 cells to stimulate IFNγ from CTLs. Staining of this CTL clone with a specific tetramer followed by flow cytometry revealed that the entire population expressed the TCR (Figure 3.8A) and secreted IFNγ following specific peptide stimulation (Figure 3.8B). About 2-3% of the clonal CSP-specific lymphocytes secreted IFNγ when stimulated with either sorted traversed or bulk infected hepatocyte cultures at 1:1 traversed hepatocyte-to-CTL ratio at different time points pi (Figure 3.8C, D). Given the clonal nature of the responder T cells, the low frequency of activated CTLs probably reflects the percentage of traversed cells capable of presenting this C-terminal CSP epitope on MHC class I and correlates with the frequencies of traversed cells harboring cytoplasmic or/and nuclear CSP (Chapter 2). Pre-treatment of sporozoites with cytochalasin D (shown to inhibit traversal, Figure 2.1) prior to infection decreased the percentage of specific CTLs secreting IFNγ nearly to the levels obtained with stimulation by non-infected cells (Figure 3.8D). To our knowledge, these data are the first direct demonstration of endogenous MHC class I-restricted antigen presentation of *P. falciparum* CSP by traversed cells.

3.3.6 CD8+ T cells limit liver stage infection *in vitro*

It has been previously described that two major cytokines released upon T cell activation, IFNγ and TNFα, can limit development of malaria parasites in the liver (Ferreira et al. 1986; Schofield et al. 1987). To determine the consequences of CSP-specific CTL activation, we assessed the ability of these T cells to limit the development of *P. falciparum* EEFs, a goal of immune responses generated in response to a liver stage vaccine. Co-incubation of YLNK-specific T cells with HC-04 cell cultures infected with *P. falciparum* 3D7-GFP parasites reduced the number of GFP+ hepatocytes by 60% and 39%, when added at 24 h and 48 h pi, respectively (Figure 3.8E). No reduction in the number of infected cells was seen in HC-04 cultures incubated with irrelevant EBV-specific HLA-A*02-restricted human CTLs. Due to technical limitations of experimental systems currently available for *P. falciparum* liver stages, we were unable to discriminate between the possible bystander effect of cytokines released by CSP-specific CTLs activated on traversed cells and the direct recognition and elimination of infected hepatocytes. However, several lines of evidence indirectly support a role for traversed cells in the control of EEF development via activation of CSP-specific CTLs. First, there are about 150 times more traversed cells present in infected cultures than EEFs, (see Figure 2.1), making them more likely to engage CSP-specific CTLs that are in close proximity to invaded cells. Second, CSP-specific CTL activation by sorted, traversed cells leads to production of IFNγ (Figure 3.8C), known to limit development of EEFs. Third, CSP can be detected in *P. falciparum* infected cells up to 4-5 days pi (Sacci et al. 2006) and, therefore, if endogenously processed and presented, should serve as a

rejection antigen within this time frame. However, our data indicate, that more efficient control of EEFs requires early, (by 24 h pi), exposure of infected cultures to CSP-specific CTLs, which are likely to recognize CSP present in and processed by traversed cells. Generation of a novel strain of *P. falciparum* parasites deficient in traversal may shed light on the role of traversed hepatocytes as regulators of parasite-specific T cells responses.

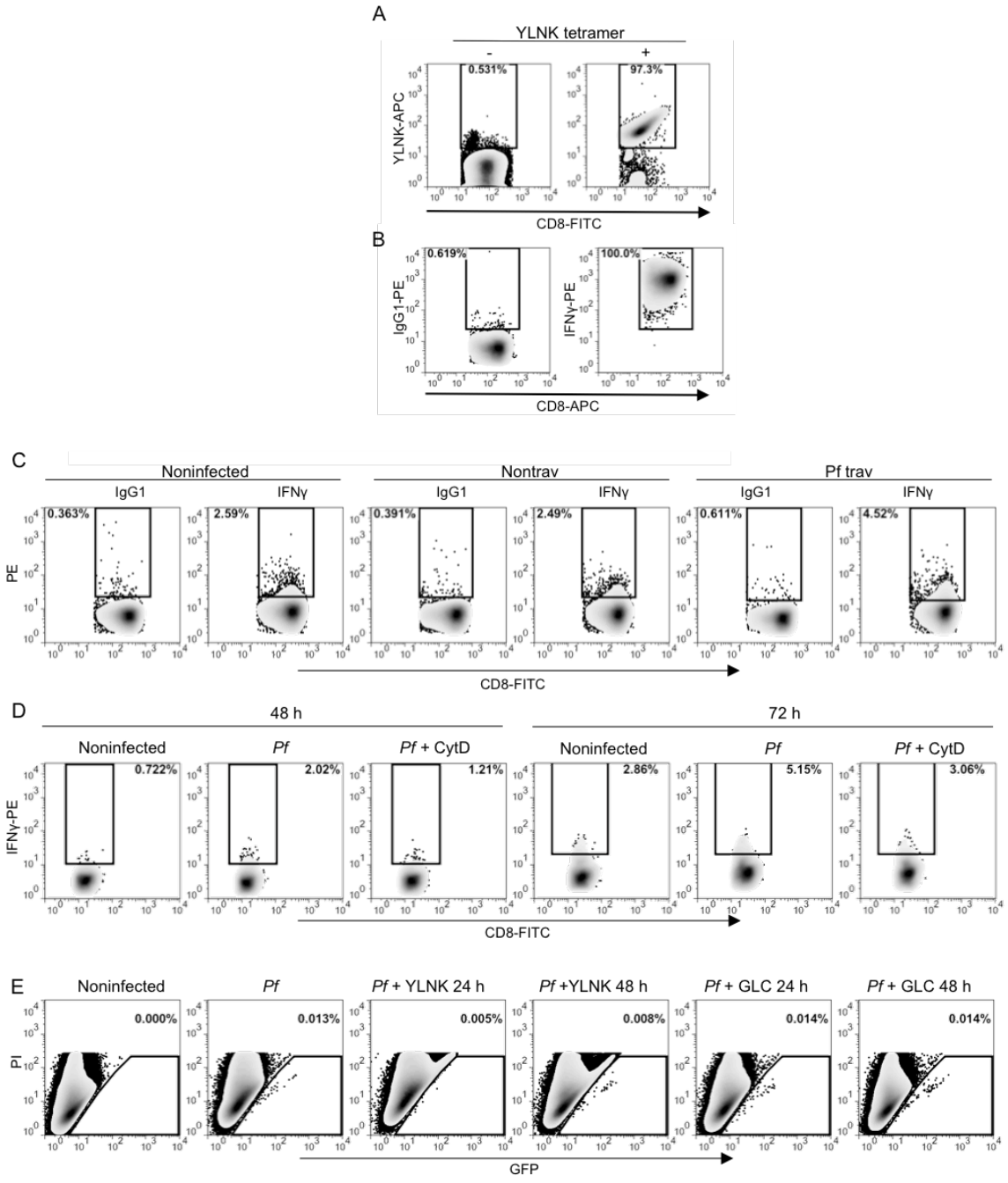

Figure 3-9. Traversed hepatocytes can activate CSP-specific CTLs.

CSP-specific CTLs recognize HLA-A*02+ HC-04 traversed with *P. falciparum* sporozoites. **(A)** Specificity of CD8+ HLA-A*02-restricted CSP-specific CTL clone was confirmed by binding to specific HLA-A*02 tetramer containing YLNKIQNSL peptide and IFNγ secretion assay following activation with the peptide pulsed targets **(B).** Numbers indicate percentages of cytokine secreting CTLs. IFNγ capture assay is described in Materials and Methods. **(C)** Representative dot plots indicate IFNγ release from CSP-specific CTLs stimulated with HC-04 cells, either uninfected or separated into traversed and nontraversed populations 24 h pi or **(D)** *Pf*-infected HC-04 cells were used as stimulators 48 and 72 h pi with sporozoites pre-incubated or not with cytochalasin D.

(E) CSP-specific CTLs can limit *Pf* infection in HC-04 cells. CSP-specific (YLNK) or control EBV-specific (GLC) CTLs were incubated with *Pf* infected HC-04 beginning at 24 or 48 h pi. Percentage of infected (GFP+) hepatocytes of viable (PI-) was determined at 72 h by flow cytometry.

3.4 Discussion

The well-established role for CD8+ T cells in the control of malaria liver stage infection suggests that liver resident cells possess *Plasmodium* antigen(s) and can serve as antigen presenting cells to trigger parasite-specific CTL responses. Currently, two subsets of hepatocytes are known to contain malarial antigens: sporozoite-traversed, and invaded hepatocytes that harbor developing EEFs. Though a number of studies suggested that infected cells can activate parasite-specific CTLs (Bongfen et al. 2007; Trimnell et al. 2009; Cockburn et al. 2013), only one has attempted to address this issue in regard to traversed cells (Bongfen et al. 2007).

In studies presented here, traversed cells were identified by dextran uptake. While we do not formally exclude the presence of invaded cells, whether harboring aborted or developing parasites, within populations of dextran-positive or dextran-negative cells, our data support a minimal role of invaded cells in T cell activation in this *in vitro P. falciparum* infection system. *P. falciparum* invaded cells are exceedingly rare as compared to traversed cells (Ch. 2), making traversed but uninvaded cells more likely to be found proximally to CTLs, and to be the source of stimulation. Therefore, to our knowledge, we present the first direct demonstration of the ability of human hepatocytes traversed with *P. falciparum* sporozoites to trigger an apparently complete program of T cell activation in an MHC class I-restricted, antigen-specific manner. Antigen presenting capacity of traversed cells was found to be equivalent to nontraversed and noninfected hepatocytes, as determined by similar surface expression of HLA-ABC (Figure 3.1).

Additionally, noninfected HC-04 cells transiently transfected to express CSP had steady-state expression of HLA-ABC and responded to pro-inflammatory cytokines to the same degree as control vector-transfected cells (Figure 3.2). Together, neither sporozoite traversal nor the presence of ectopic CSP caused obvious alterations in the immunogenicity of human hepatocytes, that contradicts a previous report (Singh et al. 2007).

CTL activation by traversed cells, manifested as release of cytokines (Figures 3.3, 3.4), upregulation of surface activation markers (Figure 3.5), degranulation (Figure 3.6) and proliferation (Figure 3.7) equivalent to that induced by stimulation with nontraversed or noninfected hepatocytes. These effects were not specific to T cell or target cell, as we utilized two different T cell clones and two targets: HC-04 cell line and primary human hepatocytes (Figure 3.4, data not shown). Interestingly, though similar numbers of T cells produced cytokines when stimulated by noninfected, traversed or nontraversed hepatocytes (Figure 3.4), higher amounts of IFNγ, TNFα, IL-2, -4, -8, and -13 were detected in co-culture supernatants when traversed cells were used a stimulators (Table 3.1). This was not due to enhanced production of these cytokines triggered by traversed hepatocytes non-specifically in antigen-independent manner (Table 3.1, no peptide), but could reflect enhanced adhesion of T cells to traversed hepatocytes due to increased expression of ICAM-1 on traversed cells (Figure 3.1).

We have shown that the proteasome is involved in the turnover of at least some CSP moieties retained by traversed hepatocytes (Chapter 2). Thus, equipped with surface HLA class I complexes and adhesion molecules (Figure 3.1), traversed cells have the potential to present parasite antigens and activate parasite-specific CD8+ T cells. We

showed that traversed HLA-A*02 HC-04 hepatocytes could indeed activate CD8+ T cells specific for an HLA-A*02-restricted C-terminal epitope of *P. falciparum* CSP (Figure 3.8). We hypothesize that other antigens, if shed into traversed cells during traversal, may activate parasite-specific CTLs in a similar fashion. If such activation by traversed cells occurs *in vivo,* we suggest several possible effects on liver stage infection.

Local production of cytokines released by activated CTLs, such as IFNγ and TNFα, which are known to limit EEF development (Mellouk et al. 1991; Depinay et al. 2011), may either directly inhibit the expansion of parasites within infected cells by inducing nitric oxide production (Sedegah et al. 1994; Klotz et al. 1995), or increase the immunogenicity of infected cells via increased expression of surface MHC class I complexes. We have previously shown that *P. berghei* infected HC-04 cells upregulated expression of genes involved in the MHC class I presentation pathway in response to treatment with activated supernatant through early time points post infection (Ma et al. 2013). Thus, traversed cells presenting antigen to *Plasmodium*-specific CTLs may improve the host control of infection.

Alternatively, traversed cells may serve as an immune decoy for parasite-specific CTLs, engaging CD8+ T cells in prolonged interactions provided by elevated ICAM-1 expression on the cell surface and deviating effectors from direct interaction with MHC class I expressing infected hepatocytes (Ma et al. 2013). However, we were not able to compare ICAM-1 expression on infected cells to that on traversed cells in these studies, as hepatocyte invasion occurs only rarely in HC-04 cells (Chapter 2).

These scenarios could be experimentally addressed in the future using genetically modified *P. falciparum* parasites incapable of traversal but competent to invade hepatocytes, such as SPECT -disrupted strains (Ishino et al. 2004).

Until now, traversal by *Plasmodium* sporozoites in the liver parenchyma has been mainly viewed as a prerequisite for efficient infection of hepatocytes preceded by the exocytosis of sporozoite apical organelles (Mota et al. 2002). In light of our findings that traversed cells maintain the ability to stimulate a full array of CD8+ T cell activation and that at least one liver stage antigen, CSP, is retained, processed, and presented, we propose a novel view on the traversed cells as a potential site of parasite specific immune activation. This presents additional questions of whether parasite-specific CTL activation by traversed cells benefit the host in controlling infection, or the parasite, in misdirecting important effector responses.

Chapter 4. DETECTION OF NATURALLY INDUCED CSP-SPECIFIC T CELL RESPONSES

4.1 Introduction

In malaria-endemic regions, populations are repeatedly exposed to and re-infected with *Plasmodium* without the developing sterile protection. Protection from clinical symptoms, but not from parasitemia, develops with age and repeated exposure, a signature of naturally acquired immunity, reviewed in (Langhorne et al. 2008; Doolan et al. 2009).

Both humoral and cellular immune responses to *Plasmodial* antigens are often detected following natural infection, but provide incomplete protection. This may be due to many factors, including insufficient effector functions or failure to generate long-term memory responses, especially considering the context of other co-morbidities such as co-infections and often observed malnutrition that affect developing regions afflicted by malaria.

Antibody responses against a broader panel of *Plasmodium* antigens develop with age and repeated exposure. These antibody profiles tend to be associated with protection from severe malaria, but do not prevent the onset of parasitemia (Barry et al. 2011; Greenhouse et al. 2011; Hoffman et al. 1987). Similar dynamics in antibody responses are also observed following periods of high malaria transmission in regions with seasonal transmission patterns, and in some cases, higher antibody levels that persist through low transmission season are predictive of less severe malaria during the next season (Crompton et al. 2010). However, the neutralizing capacity of these antibodies is poorly understood.

Studies have identified epitope-specificities and effector functions of malaria-specific T cells in naturally exposed populations. Relevant to vaccine development, MHC-class I-restricted T cell epitopes to liver stage antigens such as CSP, LSA-1, TRAP, EXP-1, and STARP (Anum et al. 2015; Offeddu et al. 2012; Lyke et al. 2005) were detected in individuals from malaria endemic regions, and there are efforts to increase the scale of these studies for antigen discovery using high throughput approaches (Grubaugh et al. 2013).

CSP-specific CD8+ T cells are often detected in peripheral blood of donors from endemic areas, albeit with low frequency (reviewed in Doolan et al. 2009). Several CD8+ T cells to specific to HLA-A*02-restricted peptides from the N- and C-termini have been detected in naturally exposed individuals, some of which may promiscuously bind to other HLA alleles (Blum-Tirouvanziam et al. 1995; Doolan et al. 1997; Wang et al. 1998; Lyke et al. 2005). Novel CTL epitopes from CSP not previously detected in the field were characterized following administration of an Adenovirus-vectored CSP-based vaccine (Sedegah et al. 2013), which may offer insight into epitopes that could be protective in vaccination that are not induced in natural exposure.

Though it is clear that naturally exposed individuals do mount humoral and cellular responses, vaccines tested in endemic populations are less effective than in naïve individuals (Guilbride et al. 2010; Cohen et al. 2010), for two possible reasons: genetic diversity in parasites that precludes efficient control of multiple circulating parasite strains, or less effective immune responses induced and maintained in the context of chronic antigenic stimulation by malarial antigens and other co-infections. Epidemiologic studies revealed alterations in the quality of adaptive immune responses in multiple

regions in different transmission settings (Butler et al. 2011; Illingworth et al. 2013; Portugal et al. 2014). Chronically exposed Malian children had features of an immunoregulatory T cell response that limited inflammation while maintaining low levels of infection (Portugal et al. 2014). B- and T-cell exhaustion have been investigated in repeated malaria exposure, manifesting as an expansion of atypical memory B cells and CD4+ T cells expressing PD-1 and LAG-3 (Illingworth et al. 2013). Increased expression of PD-1 on CD4+ T cells was detected in parasitemic Malian children, a phenomenon reproduced experimentally in mice. Furthermore, functional antibody-based blockade of PD-L1 and LAG-3 improved antibody and CD4+ T cell responses to blood stage antigens, resulting in better control of *P. yoelii* infection (Butler et al. 2011).

In our studies, we aimed to characterize the frequencies and repertoire of the MHC-I-restricted, CD8+ T cell responses to CSP in naturally exposed individuals from a high-transmission region. Using PBMCs and plasma from healthy adult donors living in Mali, we evaluated the presence of HLA-A*02-restricted CSP-specific T cell responses, measured CSP-specific IgG to each functional domain of CSP, and evaluated the effects of plasma from exposed individuals on *in vitro* infection of human hepatocytes with *P. falciparum* sporozoites. While we detected evidence of exposure (anti-MSP-1 and MSP-2 IgGs) in 78% of healthy adults and anti-CSP IgG in 62%, only 22% had detectible T cell responses to CSP as measured by IFNγ production in ELISpot assays. We found that Malian plasma samples significantly inhibited sporozoite motility as compared to plasma from non-exposed US donors. However, this inhibition neither correlated with measured anti-CSP IgG nor with inhibition of sporozoite invasion. We also found overall alteration in the composition of the T cell compartment, such as skewing of the CD4+:CD8+ T cell

ratio and increased expression of exhaustion marker PD-1. Altogether, we present a comprehensive characterization of CSP-specific humoral and cellular responses in populations from a malaria-endemic region, and the functional effects of plasma on liver stage infection. Our preliminary studies suggest a potential therapeutic benefit of PD-1 blockade for enhancement of T cell function in this population.

4.2 Materials and Methods

Ethics statement

We used human samples collected by collaborators in Mali, (Diakite Mahamadou, Malaria Research and Training Center, Bamako, Mali.) IRB approval, sample collection, processing and storage were done according to IRB protocol. De-identified samples were sent to JHU and processed as specimens with no identifiers.

Study site and sample collection

Limited amounts of peripheral blood lymphocytes and serum were collected from 45 adult volunteers at a single study site in Dangassa, Mali in December, 2012 ("ML") and June, 2013 ("AK"). Inclusion criteria were absence of clinical signs and symptoms of HIV or other acute infection and hemoglobin levels > 8.5 g/dL. Parasitemia was detected by blood smear in 4 of 20 AK donors. Samples were cryopreserved and shipped to Johns Hopkins Bloomberg School of Public Health for analysis. Ten control serum samples (further referred to as "US") from US donors with no records of previous exposure to malaria infection were provided by Dr. A. Durbin (Johns Hopkins Bloomberg School of Public Health, Baltimore, MD). "NHS" is commercially available AB Human Serum (Life Technologies, Grand Island, NY). Control "US" PBMC samples were obtained

from buffy-coats (New York Blood Center, New York, NY) and purified by density gradient centrifugation using Lymphocyte separation medium (Cellgro, Pittsburgh, PA, USA). For studies of EEF development, a smaller subset of plasma samples was randomly selected.

Detection of immunoglobulins specific to *P. falciparum* antigens by Enzyme-Linked Immunosorbent Assay (ELISA)

Plasma samples were screened by ELISA for IgGs specific for *P. falciparum* blood stage antigens (MSP-1 and MSP-2) and CSP. MSP-1 (MSPF15-R) and MSP-2 (MSP25-R) proteins were purchased from Alpha Diagnostic International (San Antonio, TX). Recombinant CSP ($rCSP_{21-382}$) was purchased from Pfenex, Inc. (San Diego, CA). The N-terminal CSP signal peptide (aa 1-20) was synthesized by the Johns Hopkins School of Medicine Sequencing and Synthesis Facility (Baltimore, MD). The central repeat region (NANP)x7 and C-terminal peptides overlapping aa 311-397 of *P.* falciparum 3D7 CSP sequences were gifts from F. Zavala (Johns Hopkins Malaria Research Institute, Baltimore, MD).

Flat-bottom MaxiSorp 96-well plates (ThermoFisher, Rochester, NY) were coated with 1 ng/µl of target antigen overnight at +4C^{o} in 0.05M carbonate-bicarbonate buffer (pH 9.6) and subsequently blocked with 1% bovine serum albumin in PBS. All plasma samples were screened at 1:100 dilutions in triplicates. Following incubation with secondary goat anti-human IgG-AP and PNPP solution (ThermoFisher) optical density (OD) was detected at 405 nm. Two thresholds of positivity were established for each individual antigen (as indicated in relevant parts of Results section): mean OD exceeding

the mean plus 2 or 3 standard deviations from the mean OD detected for each antigen in negative control samples (sera from US donors).

In vitro infection of human hepatocytes with *P. falciparum*

Human hepatocyte line HC-04 (Sattabongkot et al. 2006) was seeded to confluence in 48-well plates. *P. falciparum* 3D7HT-GFP (Talman et al. 2010) or *P. berghei* ANKA GFP (Franke-Fayard et al. 2004) sporozoites were isolated from salivary glands of *Anopheles stephensi* mosquitoes 17 or 22 days post-bloodfeed, respectively. Sporozoites were purified on OptiPrep density gradient (Sigma, St. Louis, MO) and infection procedures were done as previously described (Ma et al. 2013).

To assess sporozoite motility, traversal experiments were performed as described previously (Prudêncio et al. 2008). Sporozoites were added at 1:1 ratio in IMDM containing 100 U/ml penicillin, 100 µg/mL streptomycin, 2mM L-glutamine (Gibco, Grand Island, NY) and 1% of indicated plasma from Malian donors, control normal human serum (Life Technologies, Grand Island, NY) or Fetal Bovine Serum (Corning Cellgro, Manassas, VA) in the presence of 0.2 µg/ml of 10,000 MW dextran-tetramethylrhodamine (Molecular Probes, Grand Island, NY). For assessment of EEF development, experimental serum was either added with sporozoites simultaneously during invasion (0-48h) or 2 h later, after removal of uninvaded sporozoites (2-48h). To prevent bacterial and fungal contamination, infected hepatocyte cultures were extensively washed 2 and 24 h pi, when 5 µg/ml of Fungizone (Life Technologies) was added and experimental serum was also replenished. The percentage of cells infected with GFP-transgenic parasites was assessed by flow cytometry at 48 h pi. Only bacteria-free

hepatocyte cultures were analyzed, as determined visually and by the lack of $FSC^{low}SSC^{low}$ contaminants.

IFNγ enzyme linked immunospot (ELISpot) assays

All PBMC samples used in this study were frozen as live cell suspensions in the presence of 10% DMSO and stored in liquid nitrogen prior to analysis. Thawed PBMCs were propagated in RPMI supplemented with 10% FCS for duration of each assay. Cell viability was assessed using trypan blue. MultiScreen IP Filter plates (Millipore, Billerica, MA) were pre-wetted briefly with 35% ethanol, washed with PBS, and coated with 1 mg/ml of IFNγ-specific antibody (clone 1-D1K, MAbTech, Cincinnati, OH) overnight at 4°C. To provide conditions for possible cross-presentation of CSP, $rCSP_{21\text{-}382}$ (Pfenex) complemented by N- (aa1-20) and C- (aa 342-397) terminal CSP peptides (1 µg of total protein per well) were added directly to a portion of PBMC cultures 4 hours after thaw, whereas remaining PBMCs were left unstimulated. Twenty-four hours following thaw, ELISpot plates were washed with PBS and blocked with 10% FCS for 2 h. Aliquots containing 50,000 trypan blue-negative cells were added in duplicates for each stimulation condition. The following conditions were used to stimulate IFNγ production from PBMCs: 5 µM PHA (Sigma, St. Louis, MO), 5 µg/mL CEF peptide plus control (AnaSpec, Fremont, CA), a pool of 5 HLA-A*02-restricted viral peptides (5µM final concentration containing equal amounts of each peptide) including Influenza-derived peptides GILGFVFTL ($Matrix_{58\text{-}68}$) and FMYSDFGFI ($PA_{46\text{-}54}$), EBV-derived peptides CLGGLLTMV ($LMP2A_{426\text{-}434}$) and GLCTLVAML ($BMLF1_{280\text{-}288}$), HCMV-derived NLVPMVATV ($pp65_{495\text{-}503}$), a pool of 6 HLA-A*02-restricted CSP peptides, ($MMRKLAILSV_{1\text{-}10}$, $AILSVSSFL_{6\text{-}14}$, $HIKEYLNKI_{315\text{-}323}$, $YLNKIQNSL_{319\text{-}327}$,

GLIMVLSFLF$_{386-395}$, IMVLSFLFL$_{388-397}$)(5μM final concentration), or DMSO as a vehicle control. PBMCs from cultures stimulated with rCSP (Pfenex) complemented by N- (aa1-20) and C- (aa 342-397) terminal CSP peptides 24 h prior to assay were also plated into ELISpot plates at the same cell density. Following 20 h incubation at 37ºC and cell removal, membranes were probed with IFNγ-biotin (MAbTech), anti-biotin-AP (Vector, Burlingame, CA), and developed with BCIP/NBPT (Pierce, Rockford, IL). Spots were counted with Immunospot 5.0 (CTL, Shaker Heights, OH). Positivity for each sample was determined as number of spots exceeding the mean plus 2 standard deviations of values detected in negative control wells stimulated with DMSO.

Flow-cytometry assays for detection of surface antigens

Twenty four hours post thawing of PBMCs, antibodies specific to the following antigens were used to determine the presence of surface markers on PBMCs: CD3, CD4, CD8, (BioLegend, San Diego, CA, or BD Biosciences, San Jose, CA), HLA-A*02, clone BB7.2 (BD Biosciences) and PD-1 (BioLegend, San Diego, CA). Relevant isotype and fluorochrome control antibodies were used to determine background fluorescence in each experiment. Data were acquired with CellQuest Pro Software on FACSCalibur™ (BD) and analyzed in FlowJo (TreeStar, Ashland, OR).

Effect of allogeneic plasma on T cell subset composition and PD-1 expression

PBMCs from "negative control" US donors were incubated for 5 days with plasma samples from US and Malian subjects, at a concentration of 10 v/v% in RPMI containing 100 U/ml penicillin, 100 μg/mL streptomycin, 2mM L-glutamine, or in serum-free AIM-V medium (Gibco). Surface markers and PD-1 expression were assessed by flow cytometry as described above.

PD-1 blockade and IFNγ secretion assays

PBMCs pre-incubated with 10μg/ml of PD-1 blocking antibody or IgG1 isotype control antibody (BioLegend) were transferred into 96-well plates pre-coated with 10μg/well of anti-CD3 and anti-CD28 antibodies (BD Biosciences). For IFNγ secretion assays, following 3h incubation at 37ºC IFNγ capture was performed following manufacturer's protocols (Miltenyi Biotec, Auburn, CA). Percentage of CD4+ and CD8+ T cells secreting IFNγ was determined by flow cytometry. Alternatively, ELISpot assays were performed as described above.

Statistical Analysis

Statistical analyses were performed in Prism 6.0 (GraphPad Software, Inc., La Jolla, CA) as indicated in Figure Legends.

4.3 Results

The current study included plasma and peripheral blood from 45 healthy adults in Dangassa, Mali on the Niger River, south of Bamako, an area of high *P. falciparum* transmission (World Health Organization 2014). Previously, sample collection was conducted in December 2012 ("AK"), post-rainy season, and June 2013 ("ML"), prior to the next rainy season (Table 4.1). Ages ranged from 18 to 56, mean 37 (AK group), and 25 to 64, mean 43 (ML group). Four AK subjects were parasitemic at the time of collection. US control serum and PBMC samples included in the study were obtained from different donors.

Table 4-1. Characteristics of blood donors.

Group	ID	Age	Gender	Hb (g/dl)	Parasitemia (parasites/µl)
US					
Dec-11	A	29	M		
	B	29	M		
	C	28	M		
	D	45	F		
Jan-12	E	32	M	Not tested	
	F	20	M		
	G	27	F		
Sep-12	H	18	F		
	I	22	F		
	J	19	M		
	n=10	mean = 27	60% M (6) 40% F (4)		
AK					
Dec-12	**46**	18	F	10.4	150Tf
	47	40	F	10.8	0
	48	38	M	10	0
	49	21	M	15.5	0
	50	54	M	15	0
	51	48	M	14	0
	52	46	M	14	0
	53	38	M	17.2	0
	54	26	F	14.9	0
	55	37	M	15.7	0
	56	50	F	13.8	0
	57	28	F	10.7	0
	58	31	M	15.1	0
	59	36	M	15.7	75Gf
	60	38	M	15.6	300Tf
	61	56	F	15.2	0
	62	24	F	14.4	0
	63	35	F	11.6	0
	64	45	F	10.4	1050Tf
	65	38	F	13.7	0
	n = 20	mean = 37	50% M (10) 50% F (10)		
ML					
Jun-13	**1**	51	M	14.6	0
	2	57	M	11.8	0
	3	64	M	10.2	0
	4	45	M	15.5	0
	5	51	M	13.9	0
	6	27	F	14.4	0
	7	58	F	15.7	0
	8	40	F	14	0
	9	32	F	13.8	0
	10	28	F	14.6	0
	11	40	F	10.8	0
	12	40	F	14.4	0
	13	57	M	15.6	0
	14	45	M	15.2	0
	15	50	F	13.8	0
	16	50	F	11.6	0
	17	49	F	15.2	0
	18	51	F	14.4	0
	19	30	F	15.8	0
	20	46	M	13.7	0
	21	45	M	10.8	0
	22	30	F	14.9	0
	23	25	F	10	0
	24	29	F	15	0
	25	27	F	14.8	0
	n = 25	mean = 43	36% M (9) 64% F (16)		

4.3.1 Plasma antibodies to MSP-1 and MSP-2, CSP

As antibody responses to *Plasmodium* antigens are often detected in malaria-endemic regions, we screened plasma from healthy adult donors for IgG specific for blood stage antigens MSP-1 and MSP-2, and sporozoite/liver-stage antigen CSP. Blood stage antigens were included for evidence of infection, and antibodies against CSP were analyzed to ensure that this antigen of interest as a target of cellular immunity had been cross-presented, suggesting the possibility for T cell responses. Antibody responses to CSP were mapped to the following antigenic peptides: 1) commercially available Pfenex rCSP (aa 21-382), the full length protein excluding the N-terminal signal sequence and presumed C-terminal GPI anchor. Pfenex includes all functional domains of CSP, only omitting small portions from the utmost N- and C-termini. 2) the central repeat region represented by (NANP)x7, 3) the N-terminal signal sequence excluded from Pfenex (aa 1-20), and 4) two overlapping peptides that span the C-terminus, including the GPI anchor excluded from Pfenex (Fig 4.1A). The central repeat region contains many copies of an immunodominant B cell epitope (Zavala et al. 1983; Dame et al. 1984), while the C-termini includes multiple CD4+ and CD8+ epitopes (Doolan et al. 1997; González et al. 2000; Blum-Tirouvanziam et al. 1995). Thus, we sought evidence that regions of CSP containing T cell epitopes had been cross-presented to generate B cell responses.

ELISA assays were first performed to experimentally assess evidence of immune responses to previous *P. falciparum* infection. Plasma were screened for IgG specific for a combination of abundantly expressed blood stage antigens MSP-1 and MSP-2, as robust levels of anti-$MSP1_{19}$ IgG are induced following even a single exposure to malaria in low-transmission regions (Clark et al. 2012). Two positivity thresholds of OD

exceeding the mean plus 2 or 3 standard deviations of sera from ten healthy US donors were established, herein referred to as "2 SD" and "3 SD" thresholds. Specific OD values in thirty-two of 45 (71%) of Malian subjects exceeded the conservative 3 SD threshold, and in 35/45 (78%) of donors exceeded the more liberal 2 SD cutoff (Fig 4.1B). There was a trend toward greater percent of anti-MSP-1 and -2 positive donors in the AK group. While it was expected that nearly all subjects had experienced malaria throughout their lifetime, evidence of antibody responses exceeding our thresholds was observed in 78%.

We detected IgGs recognizing the near full-length Pfenex rCSP at the 3 SD threshold in 21/45 (47%) donors (Fig 4.1B). As expected, almost all of these subjects (19/21) had detectable anti-MSP-1 and MSP-2 IgGs (Fig 4.1B). Overall recognition of the central repeat region, represented by (NANP)x7, was detected in plasma from 22/45 donors using the conservative 3 SD threshold. All of these samples were also positive for Pfenex rCSP-specific IgG at least exceeding the 2 SD threshold. Fewer plasma samples had detectable IgG specific for the peptides including the termini, $C_{311\text{-}397}$ (9/45, 20%) and $N_{1\text{-}20}$ (3/45, 6.7%).

Both the mean OD and the percentage of donors with antibodies to all sequences of CSP were slightly higher in the AK than ML group, though these differences were not statistically significant from each other, except for the percentage recognizing the long C-terminal peptide (Fig 4.1C, D).

Most studies of antibody responses to CSP rely exclusively on screening of the central repeat region (Clement et al. 2012), which comprises 42% of the total amino acid residues. Our expanded assessment confirmed that most anti-CSP IgG are directed towards the tandem repeats, but in some cases, detectable responses are also mounted

against the termini. The functions of antibodies against each region are an important subject of future studies.

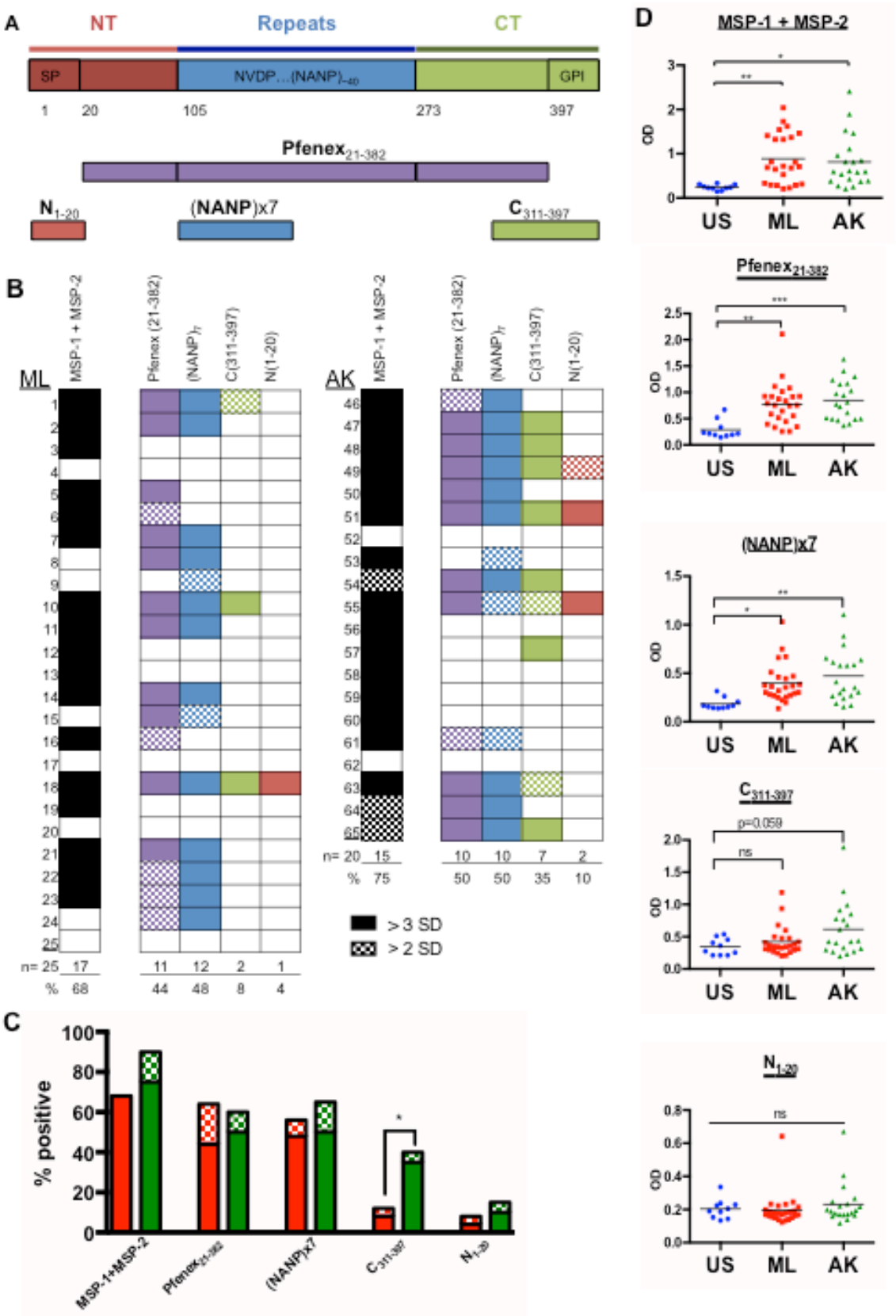
A
NT
Repeats
CT
SP
NVDP…(NANP)40
GPI
1
20
105
273
397
Pfenex21-382
N1-20
(NANP)x7
C311-397
B
ML
AK
MSP-1 + MSP-2
Pfenex (21-382)
(NANP)7
C(311-397)
N(1-20)
n= 25 17 11 12 2 1
% 68 44 48 8 4
n= 20 15 10 10 7 2
% 75 50 50 35 10
> 3 SD
> 2 SD
C
% positive
MSP-1+MSP-2
Pfenex21-382
(NANP)x7
C311-397
N1-20
D
MSP-1 + MSP-2
Pfenex21-382
(NANP)x7
C311-397
p=0.059
ns
N1-20
OD
US
ML
AK

Figure 4-1. Mapping of CSP-specific IgG responses in plasma from Malian donors

(A) Schematic of *P. falciparum* CSP functional domains with amino acid residues indicated. NT, N-terminus (red); CT, C-terminus (green), repeat region is shown in blue. Four polypeptides representing indicated CSP domains were used as capture antigens for ELISA: Pfenex $rCSP_{21-382}$ (shown in purple) N_{1-20} (red), central repeat region (NANP)x7 (blue), and $CT_{311-397}$ (green). **(B)** IgG reactivity to blood stage antigens MSP-1 and MSP-2 or indicated regions of CSP in donor plasma from ML and AK Malian cohorts were detected by ELISA in triplicates. Solid boxes indicate that the mean OD value exceeded mean + 3 SD of values obtained with control US donors. Boxes with dotted pattern signify OD values greater than mean + 2 SD but less than mean + 3 SD of values detected in control US donors. Below table, numbers (top row) and percents (bottom row) relate to samples from each cohort with OD values exceeding mean + 3 SD threshold. **(C)** Bar graph shows total percentage of samples from ML (red) and AK (green) cohorts identified as positive when tested against indicated portions of CSP. Chi-square test identified * $p \leq 0.05$ comparing number of positive samples between AK and ML groups **(D)** IgG reactivity against MSP-1, MSP-2 and 4 different CSP regions were compared in plasma of Malian and US control donors. All samples were processed simultaneously for each antigen; OD values reflecting IgG reactivity detected in each sample are shown. Horizontal lines indicate mean values of each group. One-way ANOVA followed by Tukey multiple comparison testing identified * $p \leq 0.05$, ** $p \leq .01$, *** $p \leq .001$ and **** $p \leq .0001$.

4.3.2 Plasma from Mali donors inhibits sporozoite motility

As high levels of CSP-specific antibodies have been shown to impair sporozoite motility and invasion (Mishra et al. 2012; Doolan & Martinez-Alier 2006), we investigated the ability of sporozoites to traverse HC-04 hepatocytes in the presence of plasma from malaria-exposed subjects. Interestingly, the presence of plasma from US control donors inhibited fluorescent dextran uptake by a mean of 24% over infection in the presence of FCS (Figure 4.2A). This "non-specific" inhibition of sporozoites by control US plasma, however, was significantly lower than the effect of Malian plasma, from both ML (62%) and AK (73%) groups (Figure 4.2A). This observation warrants further investigation to identify the possible "non-antibody" components circulating in human blood that can specifically impair the migration of *P. falciparum*, but not *P. berghei* sporozoites through human hepatocytes (Figure 4.2B).

While specific for *P. falciparum,* the inhibition of sporozoite motility was independent of the presence or the magnitude of Pfenex rCSP-specific IgG (Figure 4.2C, D). Plasma from Malian donors, regardless of the levels of anti-CSP antibodies in peripheral blood, strongly limited sporozoite traversal as compared to US plasma. Furthermore, there was no statistical correlation observed between OD values for antibodies recognizing each CSP peptide and inhibition of traversal in the US or ML groups (Figure 4.2D). Surprisingly, plasma from AK subjects had a statistically significant, though weak, negative correlation between the inhibition of traversal and antibody titers to "full length," (NANP)x7, and C-terminal regions of CSP, (Figure 4.2D).

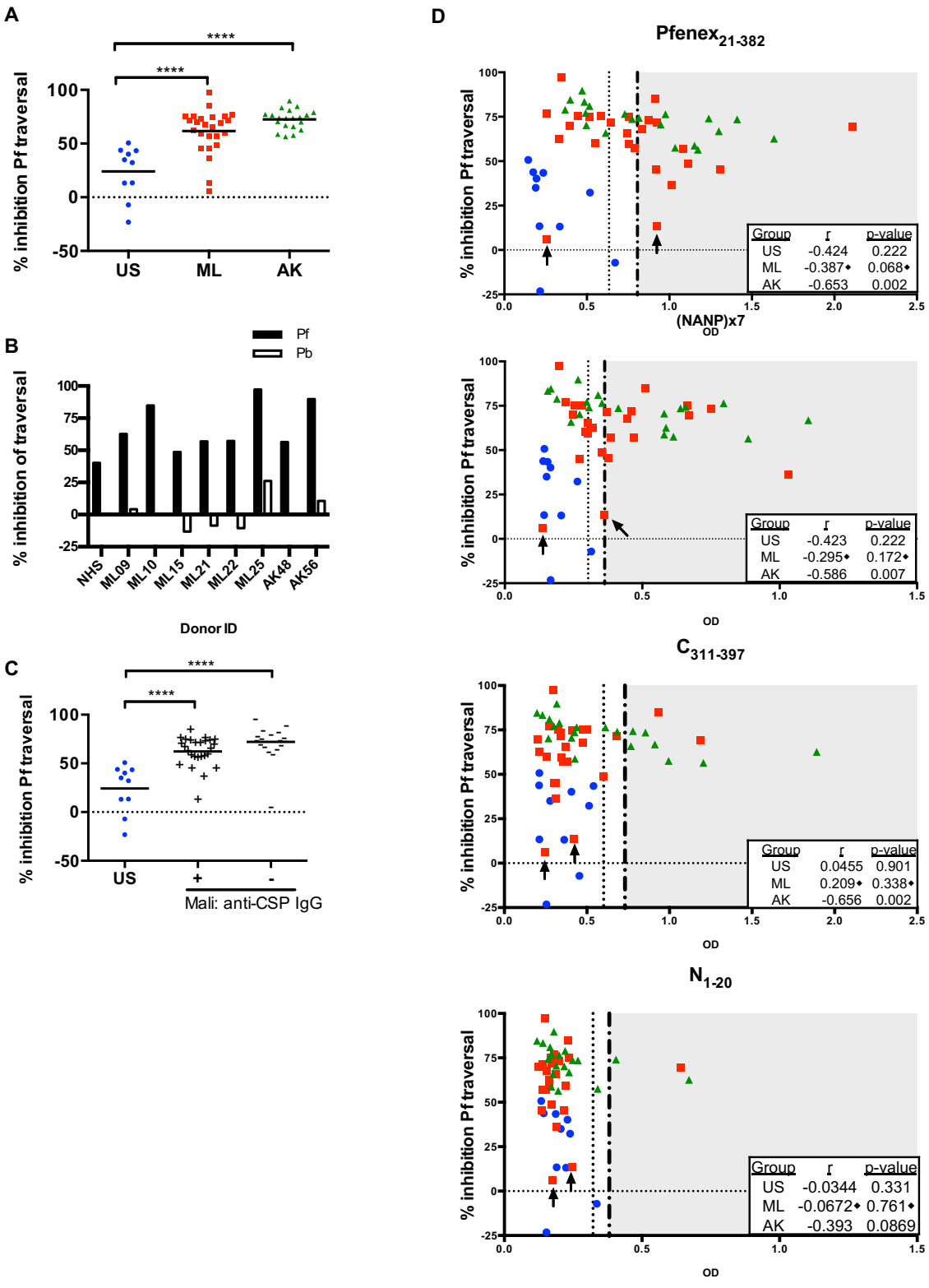

Figure 4-2. Effect of plasma from Malian donors on sporozoite motility *in vitro*

Motility of *P. falciparum* sporozoites was assessed by *in vitro* traversal assays as described in Materials and Methods. **(A)** Comparison of traversal inhibition by plasma from ML and AK Malian cohorts and US control plasma samples. Traversal assays are described in Materials and Methods. Inhibition of traversal was calculated as percentage of traversed hepatocytes detected in the presence of Malian or US control plasma relative to that detected in the presence of FCS. **(B)** Comparison between the ability of randomly selected Malian plasma samples to inhibit *P. falciparum* and *P. berghei* sporozoite motility, measured in traversal assays. **(C)** Comparison of traversal inhibition by plasma from either US control or Malian donors (ML and AK cohorts combined) separated into samples with IgG reactivity to $Pfenex_{21\text{-}382}$ either above (+) or below (-) 3 SD threshold. Horizontal lines **(A,C)** indicate mean values of each group. One-way ANOVA followed by Tukey multiple comparison testing identified * $p \leq 0.05$, ** $p \leq .01$, *** $p \leq .001$ and **** $p \leq .0001$. **(D)** Correlation between the IgG reactivity against indicated antigenic regions of CSP detected in plasma of Malian or US control donors and inhibition of traversal. Y-axes represent the decrease in percentages of traversed hepatocytes detected in the presence of plasma from US control or Malian donors, as compared to percentage of traversed hepatocytes observed in the presence of FCS, arbitrarily taken as 100%. X-axes indicate OD values for individual plasma samples obtained from ELISA screening experiments (as described in Figure 4.1). Individual plasma samples are denoted by blue circles (US controls), red squares (ML) or green triangles (AK). Vertical dotted line and dashed/dotted indicate thresholds of 2 and 3 SDs above mean of values obtained with control plasma samples of US donors. Grey shading indicates samples with OD values exceeding mean + 3 SD threshold. Inserted tables show correlation (r-value) between OD and traversal inhibition for each group of samples and statistical significance (p-values), both calculated using GraphPad Prism correlation function. "Diamond" symbols in table and arrows on scatterplot indicate 2 outliers from ML group as defined by Grubb's outlier test, which were removed from correlation calculations.

4.3.3 Effect of plasma on *in vitro* infection of human hepatocytes

Sporozoite traversal reflects the capacity of sporozoites to migrate through hepatocytes and can be used to assess sporozoite motility *in vitro*. Productive infection, however, requires both invasion and successful establishment of the parasitophorous vacuole (PV) followed by replication within. Therefore, we assessed the effect of human plasma on the development of *P. falciparum* 3D7HT-GFP EEFs, measured as the percent of GFP+ cells 48h pi. HC-04 hepatocytes were infected with *P. falciparum* sporozoites either simultaneously with or prior to addition of plasma. Overall, the presence of Malian plasma during sporozoite infection controlled EEF development versus normal human serum, this effect, however, was not statistically significant (95% CI 0.0127% - 0.0269%, NHS mean = 0.0234%, p = 0.158) (Figure 4.3A) and rather reflected the findings that

treatment with only some of AK plasma samples, such as AK48 and AK56, led to a decrease in the number of infected cells.

To the contrary, when plasma was added 2 h pi, after removing uninvaded sporozoites, EEF development was significantly enhanced overall compared to normal human serum control, though to a small degree (95% CI 0.02789% – 0.03860%, NHS mean= 0.0244%, p = 0.0058). This demonstrates, that a large pool of Malian plasma samples facilitated development of EEFs when used under experimental conditions precluding direct effects on sporozoites prior to invasion. This time-dependent effect of plasma is illustrated in Figure 4.3B. Interestingly, AK48 and AK56 plasma exhibited the greatest difference in their opposing effect when added at 0 h (decrease in %GFP+ cells) or 2 h (increase in %GFP+ cells) pi.

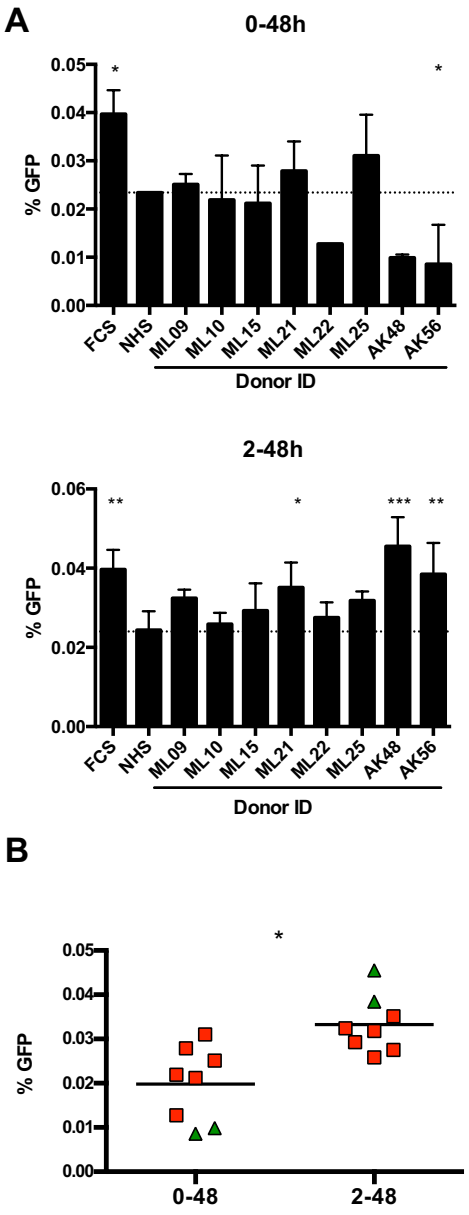

Figure 4-3. Effect of plasma from Malian donors on *P. falciparum* EEF development *in vitro.*

(A) HC-04 cells were infected with *P. falciparum* 3D7-HTGFP in the presence of plasma from indicated donor added either simultaneously with sporozoites (0-48) or 2 h pi (2-48) after cells were washed and uninvaded sporozoites were removed. The percentage of infected GFP+ HC-04 cells was determined by flow cytometry 48 h pi. Dotted line indicates level of infection observed in hepatocyte cultures infected in the presence of normal human serum (NHS). Values * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ were calculated using one-way ANOVA, and Fisher's LSD to compare effect of plasma from indicated Malian donor and commercial NHS. **(B)** Comparison of effect of each plasma sample on infection rate (%GFP) when added to HC-04 cells simultaneously with sporozoites (0-48) or beginning 2 h pi (2-48). ML, red squares; AK, green triangles. Value * $p < 0.05$ is identified by paired t-test between "0-48" and "2-48" groups of samples.

4.3.4 T cell composition

The viability of cells recovered following thaw for each donor ranged from 0 to 80% as measured by trypan blue exclusion. The number of recovered cells ranged from 0 - 10^6 per donor. Thirty-eight out of 45 (84%) donors had sufficient viable PBMCs to perform ELISpot and flow cytometric analysis of T cell composition and HLA-A*02 status. The size of CD4+ and CD8+ T cell compartments in PBMCs from Malian donors varied significantly from that of control US donors (Fig 4.4A). Within CD3+ lymphocytes, Malian samples had lower percentages of CD4+CD8- (Mali mean 38.1%, US mean 66.0%), higher percentages of CD8+CD4- (Mali mean 44.7%, US mean 20.3%), significantly higher percentages of CD4+CD8+ (Mali mean 11.5%, US mean 2.79%), and significantly lower percentages of CD4-CD8- (Mali mean 5.78%, US mean 10.9%) cells compared to US samples, though the high amount of double negative T cells in the US controls was skewed by two outliers. Skewed CD4+:CD8+ ratios were observed in PBMCs from Malian donors (Mali mean 0.997, US mean 3.30). While the mean ratio was strongly affected by outliers in the US donors, the median ratio the median also indicates that the CD4+:CD8+ ratio in PBMCs from Malian donors was lower than those from the US.

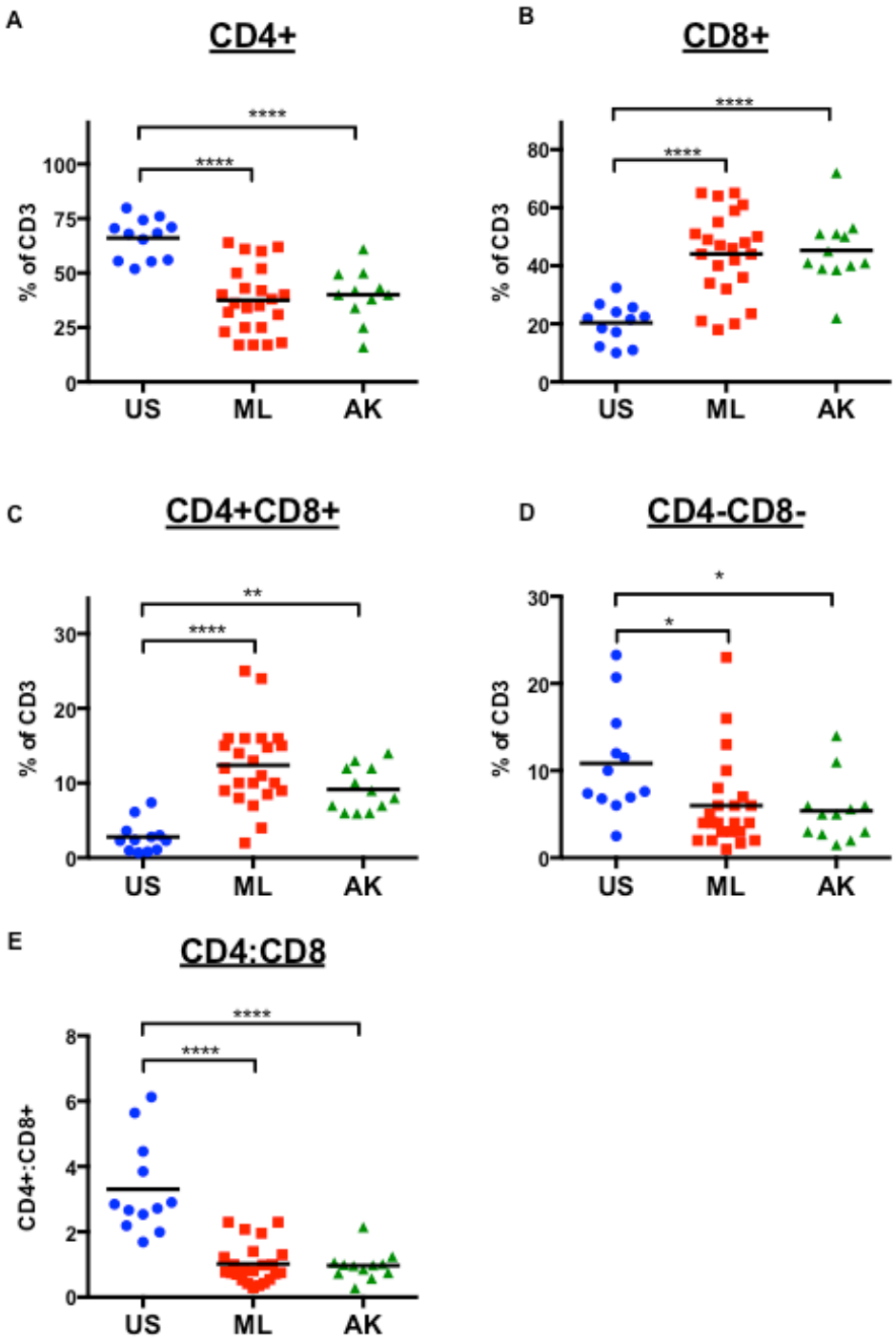

Figure 4-4. Lymphocyte subsets in PBMCs from Malian donors.

The following subsets of CD3+ lymphocytes were quantified by flow cytometry in PBMCs from US control donors or Malian donors from AK and ML cohorts: CD8-CD4+ (A), CD8+CD4- (B), CD8+CD4+ (C) and CD4-CD8- (D). CD4:CD8 ratio for each group of donors is shown in (D). Values * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ were obtained using one-way ANOVA followed by Tukey multiple comparison test.

4.3.5 IFNγ production in PBMCs following stimulation with viral and CSP-derived peptides

PBMCs from Malian donors were stimulated *ex vivo* to determine the presence of CSP-specific memory T lymphocytes. Using a positivity threshold of 2 standard deviations above the mean number of spots for all control DMSO-stimulated samples, we found that PBMCs from 16/38 (42%) of Malian donors produced IFNγ following stimulation with a control pool of viral HLA class I-restricted peptides CEF (Figure 5A, B). This is far lower than the published 88% of expected responders (Currier et al. 2002). This could be due to a mismatch between the HLA haplotypes of donors' PBMCs used in our study and the MHC restrictions of viral epitopes included in CEF, low amount of responding PBMCs used in our assay, as only 40,000 – 50,000 PBMCs were plated per well due to limited numbers available, or broad immune suppression in T cell compartment of Malian donors.

Two methods were used to determine CSP-specific IFNγ responses: stimulation with Pfenex rCSP to induce cross presentation, and with selected HLA-A*02-restriced 9- and 10-mer peptides from CSP. Stimulation with Pfenex rCSP yielded IFNγ production in 6/38 (15.7%) of donors when 3 SD threshold positivity approach was applied for analysis, and in 8/38 (21%) of donors using 2 SD threshold (Figure 4.5 A, B). Four of the six "high responders" also responded to CEF, but two did not (ML24, AK58). This suggests that these two "CEF non-responders" were indeed capable of antigen-specific stimulation, but may not have recognized the CEF peptides.

Twelve of 38 (32%) donors were HLA-A*02-positive as determined by flow cytometry (Figure 4.5A). However, the anti-HLA-A*02 monoclonal antibody BB7.2 has been shown to bind HLA-A*69, -A*68, -A*23, and -A*24 as well (Hilton & Parham 2013). Therefore, this may be an overestimate of the frequency of HLA-A*02 in our study, which was determined to be only 21% of the larger Malian population under study (Cao et al. 2004). As an alternative determination of the HLA-A*02 haplotype and presence of functional HLA-A*02-restricted CD8+ responses, PBMCs were stimulated with only the HLA-A*02-restricted viral peptides contained within CEF ("Viral A2"). The number of responders was the same as to Pfenex rCSP (Figure 4.5B). Four of six samples that responded to the viral HLA-A*02-restricted peptides were also HLA-A*02+ by flow cytometry. The PBMCs that were defined as HLA-A*02 negative could bind the selected peptides on cross-reactive HLA alleles. Four of six responders to viral HLA-A*02-restricted peptides also responded to CEF control peptides. The total amount of protein used for stimulation with CEF and the Viral A2 peptides was identical; therefore, a greater amount of each individual peptide was included in the Viral A2 pool, possibly explaining the absence of IFNγ production upon stimulation with CEF.

CSP epitopes were chosen based on documented binding to HLA-A*02, IFNγ production or cytolytic activity following stimulation in naturally exposed or immunized volunteers, or predicted binding (Blum-Tirouvanziam et al. 1995; Doolan et al. 2000; González et al. 2000; Sedegah et al. 2013). Four of 38 PBMCs samples responded to the CSP-derived HLA-A*02-restricted peptide pool: ML11, ML22, AK47, and AK51 (Figure 4.5A,B). PBMCs from donors ML11 and AK47 responded to all stimulation conditions and expressed surface HLA-A*02. Therefore, these donors may be of utility

for the generation and expansion of CSP-specific CD8+ T cell lines for *in vitro* studies of presentation and cytotoxicity in infected or traversed hepatocytes.

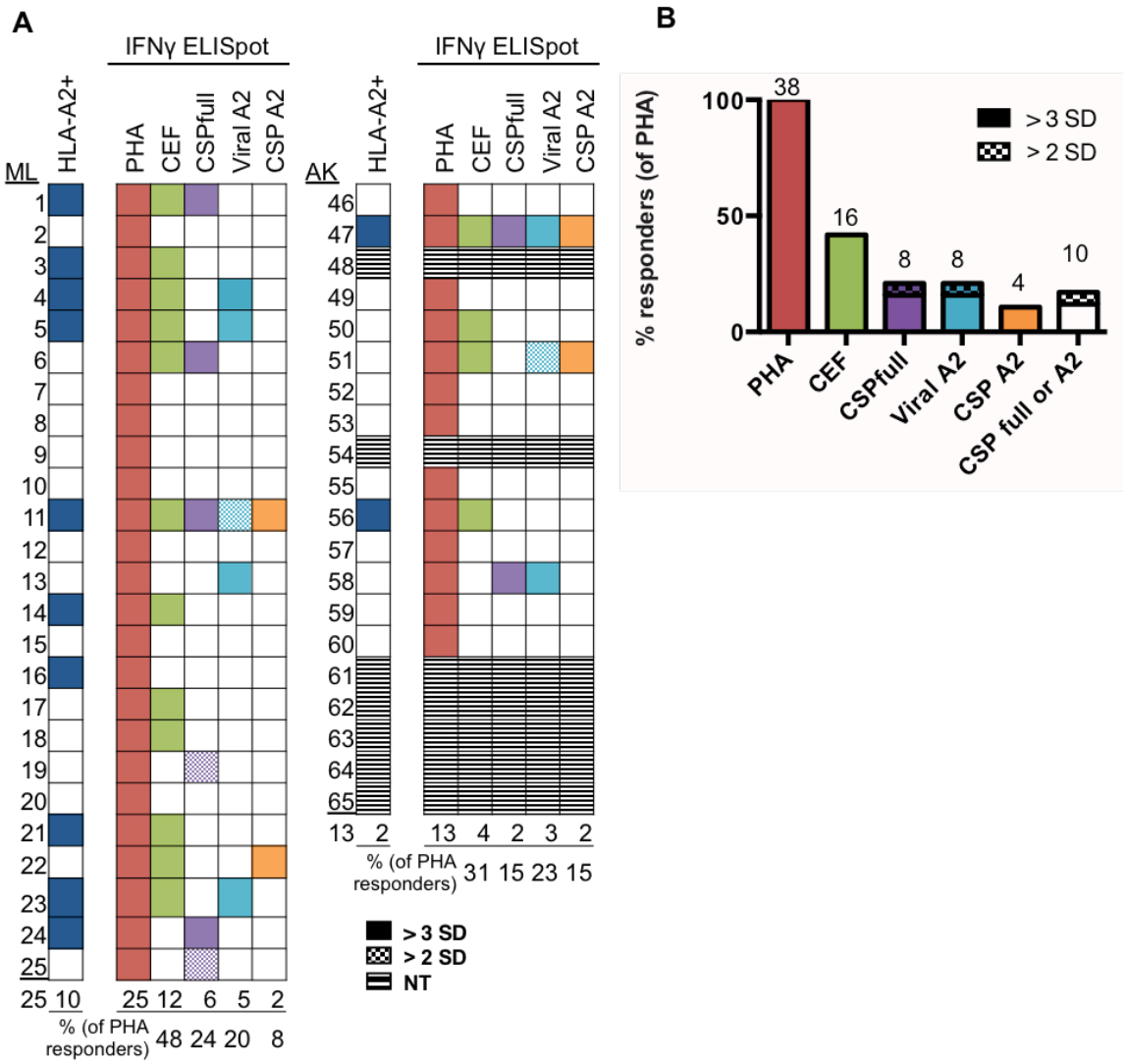

Figure 4-5. Detection of CSP-specific T cell responses in PBMCs from Malian donors.

(A) IFNγ responses were detected by ELISpot assays following stimulation of PBMCs with PHA, CEF control peptide pool, peptides spanning the full length of CSP (CSPfull), HLA-A*02-restricted viral peptides (Viral A2), or HLA-A*02-restricted peptides derived from CSP (CSP A2). Solid boxes indicate samples with number of spots exceeding 3 SD above the mean value derived from all unstimulated control samples propagated in DMSO (vehicle control). Dotted boxes indicate samples with number of spots exceeded 2 standard deviations (as described above). Horizontal lines indicate PBMC sample that was not tested (NT) due to insufficient cell viability to perform assay. Values below tables indicate number of responders to each antigenic stimulus, using cutoff above 2 SD of DMSO control (top row) and percent of responders to each specific stimulus relative to

a number of PHA-responders (bottom row). HLA-A*02 status is indicated for each PBMC sample (black boxes). **(B)** Bar graph summarizes the percent of responders to each antigenic stimulus in ML and AK Malian cohorts combined relative to the number of all PHA-responders.

4.3.6 Increased PD-1 expression in Malian PBMCs

To possibly explain the infrequent overall responses to viral peptides in Malian samples, PBMCs were stained for inhibitory signaling receptors PD-1 (Figure 4.6) and Tim3 (data not shown). While surface expression of Tim3 was undetectable, PD-1 expression was significantly increased in Mali versus US control PBMCs (Figure 4.6A). The most notable increase in PD-1+ cells was within the CD4+CD8- and CD4-CD8- populations (Figure 4.6A,B). The majority of double positive, CD4+CD8+ T cells in PBMCs from both populations were PD-1+. However, this population comprises a more substantial portion of Malian versus US PBMCs (Figure 4.4C). Overall, increased PD-1 expression in the T cell compartment suggests a broad regulatory phenotype within Malian PBMCs not limited to malaria-specific T cells.

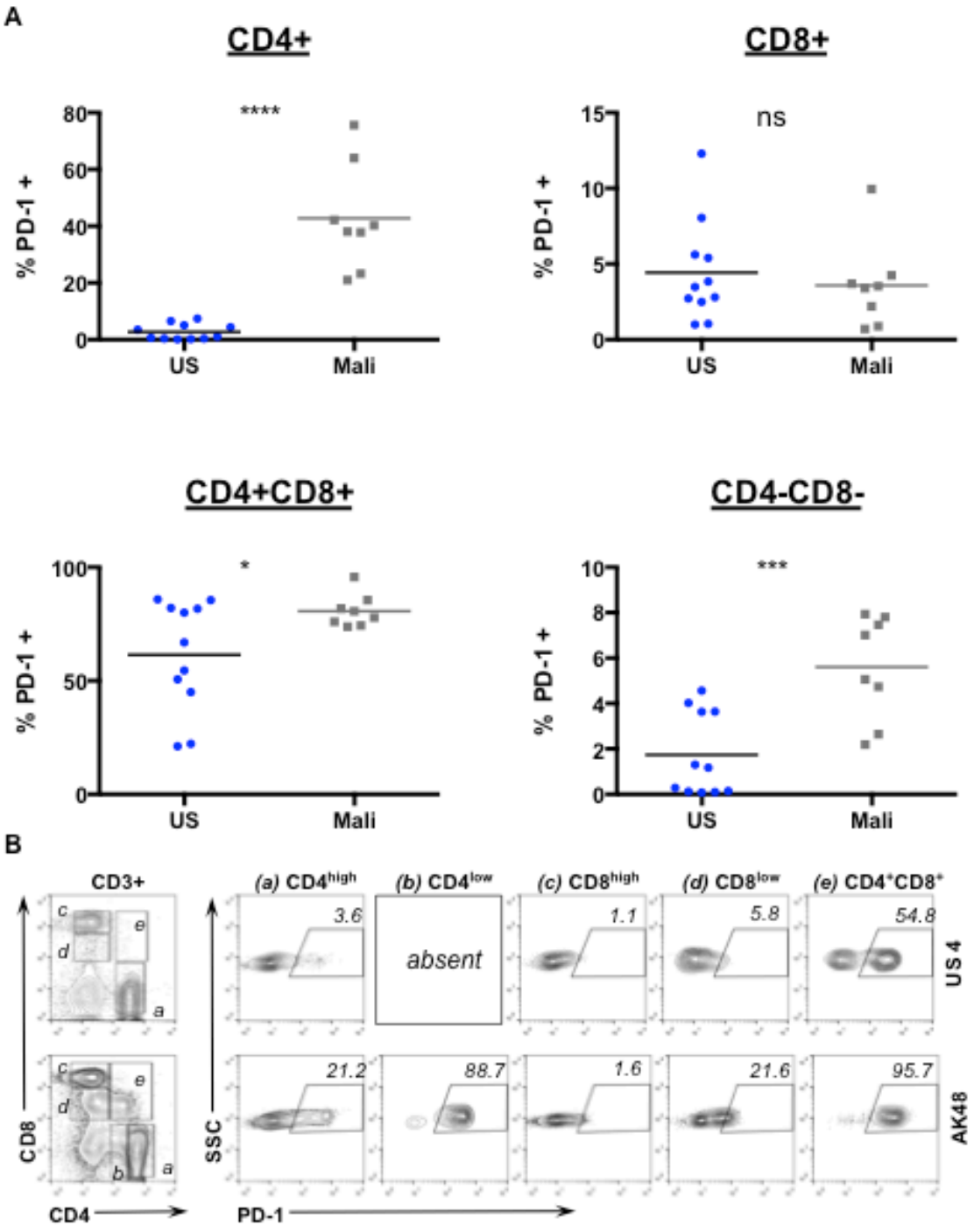

Figure 4-6. PD-1 expression on T lymphocytes from Malian and US donors

(A) Percentage of PD-1 positive cells within each indicated T cell subset of US and Malian donors was determined by surface staining and flow cytometry. Values * $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$ were obtained using student's T-test. **(B)** Representative FACS plots illustrating PD-1 expression within each CD8+ and CD4+ subsets (a-e) in control PBMCs from donor US4 and Malian PBMCs AK48.

4.3.7 *Trans*-effect of Malian plasma on malaria-naïve PBMCs

PBMCs from US donors were propagated in the presence of plasma from allogeneic US or Malian donors to investigate if the altered T cell subsets and PD-1 expression seen in Malian PBMCs could be induced in *trans*. Only one of three malaria-naïve PBMC samples tested, (US 3) had a tendency toward altered CD4+:CD8+ ratio following treatment with Malian plasma (Figure 4.7). However, these differences, though statistically significant, were subtle and did not match those seen in PBMCs from Malian donors. Interestingly, incubation of US3 PBMCs in the presence of Malian plasma significantly increased the pool of CD4-CD8- cells (Figure 4.7). However, a functional role for these alterations is unclear.

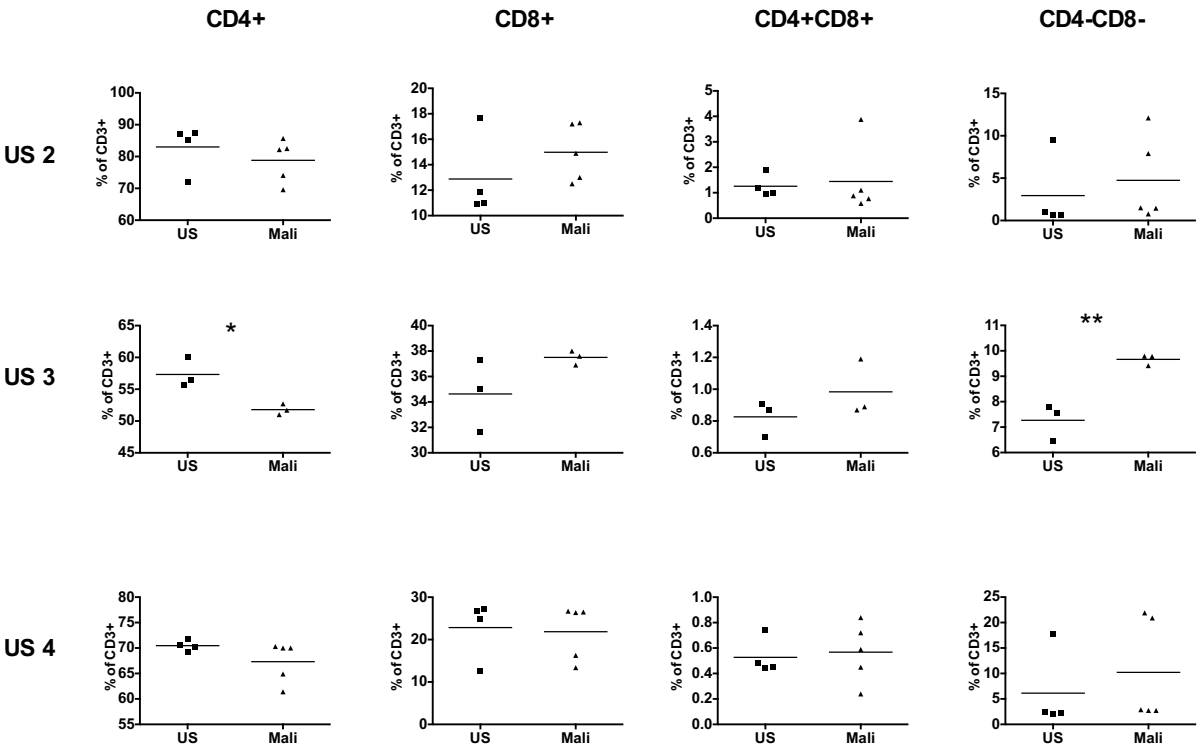

Figure 4-7. Trans-effect of plasma from Malian donors on T cell composition in PBMCs from US donors

PBMCs from three US control donors (US2, US3, US4) were propagated for five days in either serum-free AIM-V medium or AIM-V medium containing 10v/v% of plasma from US control or Malian donors. T cell subset composition was assessed by flow cytometry as described in Materials and Methods. Scatter plots indicate CD4+CD8-, CD8+CD4-, CD4+CD8+, and CD4-CD8- subsets as percentages of total CD3+ cell pool found in each indicated "normal" PBMC sample upon different treatment conditions. Value * $p \leq 0.05$, ** $p \leq 0.01$ was obtained using student's T-test.

Next, we assessed the ability of plasma from Malian donors to regulate PD-1 expression on PBMCs. Again, each PBMC donor exhibited different responses to US and Malian plasma (Figure 4.8). CD4+ and CD8+ T cells from Donor 3 slightly increased PD-1 expression when exposed to Mali serum, and US 4 had a reduction in PD-1+ cells, but these differences did not exceed one percent. These studies suggest that circulating plasma factors in healthy adults from Mali likely do not exhibit biologically meaningful alterations to CD4+ and CD8+ T cell frequencies or inhibitory PD-1 expression.

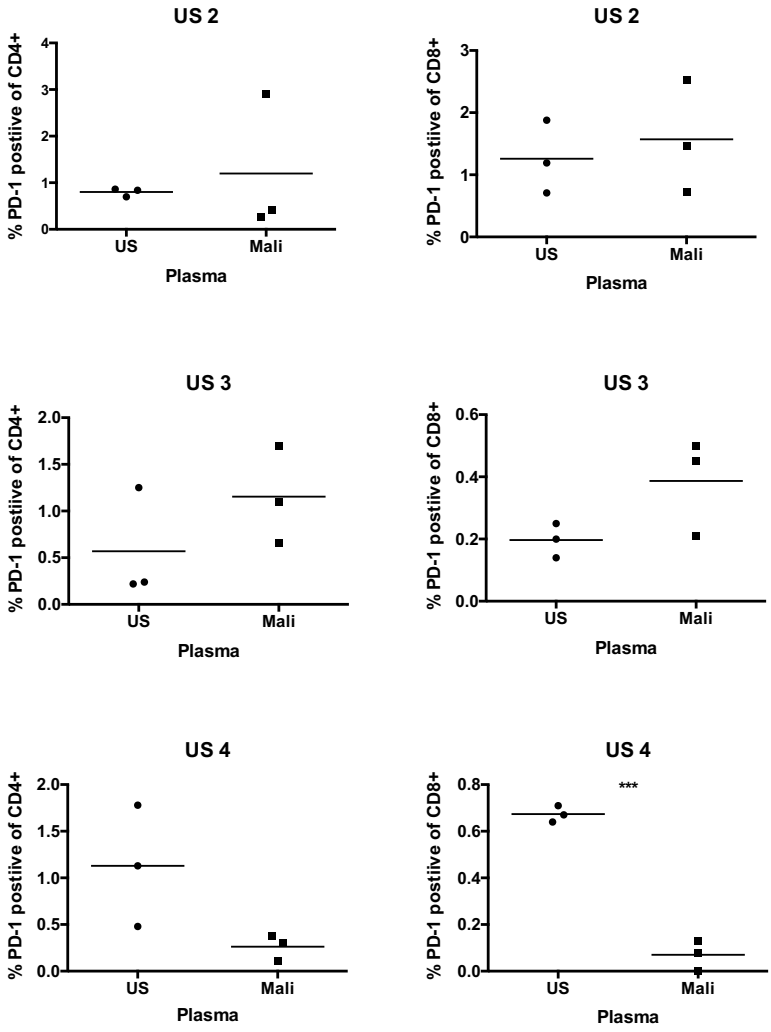

Figure 4-8. Trans-effect of plasma from Malian donors on PD-1 expression by PBMCs from US donors

PBMCs from three US control donors (US2, US3 and US4) were propagated for five days in either serum-free AIM-V medium or AIM-V medium containing 10v/v% of plasma from randomly selected US control or Malian donors. PD-1 expression within each indicated T cell subset was determined by flow cytometry. Scatter plots indicate percentage of PD-1 positive cells of CD3+ lymphocyte subsets (left, CD4+; right, CD8+) found in each indicated US PBMC sample upon different treatment conditions. Value *** $p \leq 0.001$ was obtained using student's T-test.

4.3.8 PD-1 blockade increases response of Malian PBMCs to T cell receptor triggering

To assess a function associated with the observed increased PD-1 expression by Malian PBMCs, a pilot study was performed in which PBMCs from one Malian donor

were subject to antibody-based PD-1 blockade prior to TCR triggering with anti-CD3 and anti-CD28 stimulation. In donor AK48, PD-1 blockade increased the percent of CD4+ cells secreting IFNγ from 4.67% to 7.68%, and from 57.5% to 64.8% of CD8+ cells. This pilot test of PD-1 blockade shows promise for enhancing cytokine secretion from T cells, but these findings need to be expanded to other donor PBMCs and to antigen-specific stimulation.

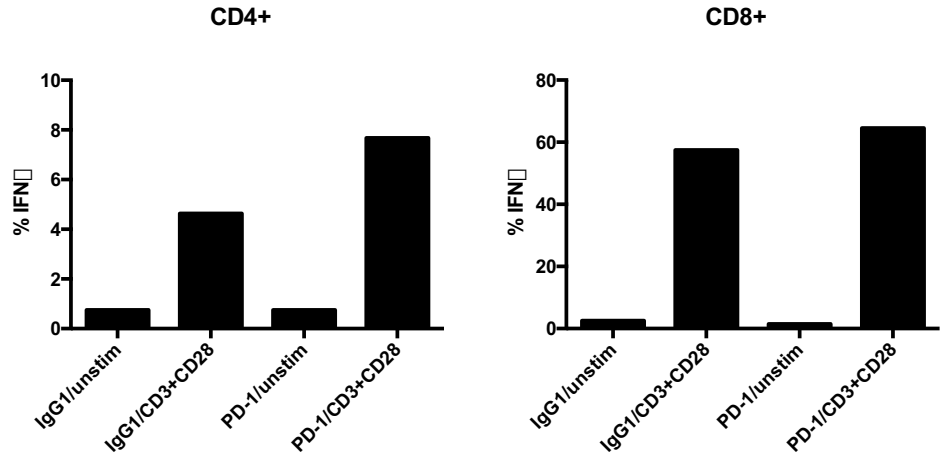

Figure 4-9. Effect of PD-1 blockade on response to TCR triggering of PBMCs from Malian donor.

PBMCs from Malian donor AK48 incubated overnight with PD-1 blocking antibody or IgG1 isotype control antibody were stimulated with anti-CD3 and anti-CD28 beads followed by detection of secreted IFNγ by flow cytometry. Bar graphs indicate percentages of CD4+ and CD8+ cells secreting IFNγ.

4.3.9 PD-1 blockade increases response of Malian PBMCs to CSP-derived, MHC-I-restricted peptides

Next, we sought to determine if interfering with PD-1-based interactions of T cells with APCs could modulate malaria-specific CD8+ T cell responses. We selected HLA-A*02-positive donors with previously undetectable IFNγ production following stimulation with CSP-derived peptides in ELISpot (Figure 4.5) and stimulated in the

presence of PD-1-specific (or isotype control) antibodies. PD-1 blockade in two of three Malian donors tended to increase the number of cells secreting IFNγ following stimulation with CSP-specific HLA-A*02-restricted peptide pools, though not statistically significantly (Figure 4.10). These data will be extended to additional PBMC donors in the future to assess the functional effects of increased PD-1 expression by T cells.

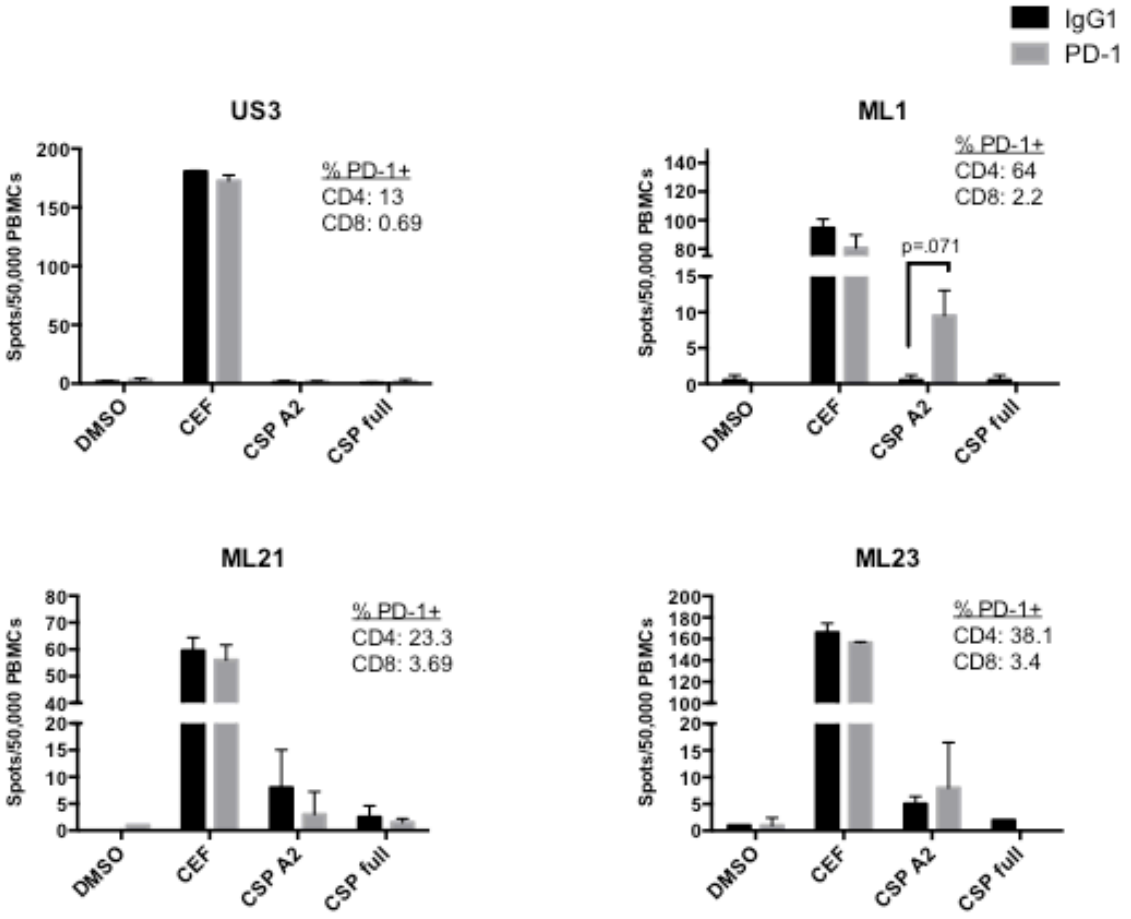

Figure 4-10 Effect of PD-1 blockade on response to CSP-specific, MHC-restricted stimulation of PBMCs from Malian donors.

PBMCs from US control and Malian donors were stimulated with viral control or CSP-specific peptides for 20 h in the presence of PD-1 blocking antibody or IgG1 isotype control antibody, followed by detection of IFNγ-producing cells by ELISpot. Bar graphs indicate mean number of spots and standard deviation per 50,000 PBMCs stimulated in duplicate.

4.4 Discussion

To understand the nature of CSP-specific memory T cell responses induced during natural infection with malaria, we analyzed plasma and PBMC recall responses in healthy adults from Mali. We first assessed evidence of exposure to *P. falciparum*, as IgG against blood stage antigens MSP-1 and MSP-2. It is documented that ninety percent of populations living in malaria-endemic regions of Mali are exposed to multiple infectious mosquito bites each season (World Health Organization 2014). In line with this observation, we found evidence of humoral responses to MSP-1 and MSP-2 in 78% of the 45 subjects in our study (Figure 4.1B). While it was expected that the entire population samples would have experienced malaria illness during childhood and been exposed each season, antibody titers are associated with current or recent blood stage infection (Proietti et al. 2013). Accordingly, 90% of the post-transmission season AK cohort, and 68% of the pre-transmission season ML cohort had detectable titers to MSP-1 and MSP-2 (Figure 4.1B).

With evidence of previous malaria infection, we investigated the presence CSP-specific IgG. Most studies screening for CSP IgG by ELISA solely rely on peptides of the repeat region (Clement et al. 2012). Because we were primarily interested in assessing CTL responses to previously identified peptide epitopes derived from the N- and C-termini, we expanded these methods to assess humoral responses against the termini by including a nearly full length recombinant protein, and peptides spanning the N-terminal signal peptide and C-terminus that were not included in the commercially available full-length recombinant CSP (Figure 4.1A). As the central repeat region is recognized to

contain an immunodominant epitope of CSP (Zavala et al. 1983; Dame et al. 1984), it was not surprising that the number of subjects recognizing the more inclusive Pfenex rCSP and (NANP)x7 peptides were nearly identical. Nearly all donors with IgG specifically recognizing the (NANP)x7 peptide indeed were also positive for IgG against Pfenex rCSP (Figure 4.1B). Fewer plasma samples had IgG specific to the abbreviated N- and C-termini tested, and the number of positive samples tended to be higher in the post-season AK group (Figure 4.1C). This finding is in agreement with other studies showing that anti-malaria antibodies are short-lived and increase after each transmission season, though these documented differences are greater in children than in adults (Crompton et al. 2010).

Since we detected antibody responses to CSP, which is abundantly expressed on the sporozoite surface, we next assessed the functional effect of Mali plasma samples on *in vitro* infection of hepatocytes. As compared to infection in the presence of FCS, the addition of human plasma during invasion, whether from exposed or non-exposed individuals, inhibited sporozoite motility (Figure 4.2A). This inhibition, as measured by traversal, was specific to *P. falciparum* and not the rodent parasite, *P. berghei*. However, this inhibition did not correlate with the presence or magnitude of anti-CSP antibodies measured by ELISA (Figure 4.2C, D). Interestingly, this finding differed from other reports indicating that inhibition of motility correlated with the concentration of monoclonal anti-CSP antibodies or IgG purified from vaccinated human subjects (Foquet et al. 2013; Behet et al. 2014; Finney et al. 2014). However, since vaccines are designed to deliver antigen in an optimally immunogenic manner, inducing sufficiently high amounts of memory B cells, the function of vaccine-induced antibodies may have greater

neutralizing capacity than those induced in natural exposure. Thus, the possible component(s) of plasma beyond CSP-specific antibodies from Malian donors that impair motility of sporozoites *in vitro* have yet to be identified. In the future, we intend to deplete IgG from these plasma samples to see if this effect is antibody-mediated. The specificity for impairment of *P. falciparum* versus *P. berghei* sporozoite motility suggests an antigen-specific plasma factor.

We also assessed the effects of human plasma on EEF development, using a smaller panel of randomly selected samples. We observed an overall decrease in percentage of GFP-positive cells when adding plasma during sporozoite invasion, and an increase when adding plasma post-invasion (Figure 4.3A,B). However, these findings should be repeated using a greater number of samples to understand this phenomenon.

With experimental evidence that the PBMCs from Malian donors had been exposed to and developed humoral responses against CSP, we next assessed T cell responsiveness. We characterized the composition of CD4+ and CD8+ subsets of the CD3+ compartment in peripheral blood, and observed lower CD4+:CD8+ ratios and a higher percent of CD4+CD8+ cells, as compared to US control donors (Figure 4.4). We then screened for responses to control viral peptides, full-length CSP, or selected HLA-A*02-restricted peptide epitopes derived from CSP by IFNγ ELISpot.

We observed lower than the expected population-wide 89% of responses to viral peptide pool CEF (Currier et al. 2002) (Figure 4.5). This could be due to many reasons, primarily 1) 50,000 PBMCs were used per stimulation due to poor viability and availability of the specimens, less than ideal for detection of rare antigen-specific cells or 2) immune dysregulation affecting T cell responsiveness. Mali is one of the poorest

countries in the world, burdened by malnutrition and many other infections besides malaria, which can alter adaptive immunity. Chronic exposure to malaria is associated with both B- and T-cell exhaustion, with strong implications for inhibitory T cell signaling receptor, PD-1 (Weiss et al. 2009; Illingworth et al. 2013; Butler et al. 2011). Therefore, we investigated the expression of PD-1 on PBMCs, and if exposure of PBMCs from malaria-naïve control US donors to Malian plasma could alter expression of CD4, CD8, and PD-1. We found increased expression of PD-1 on PBMCs from Malian donors, mostly observed in the population of double positive, CD4+CD8+ cells that was more prominent in Malian donors (Figure 4.6). This population of peripheral double positive cells is rarely seen in healthy individuals, but is detected in several conditions associated with immune deficiency, such as HIV, aging, and chronic viral infection or parasitic infection, and are often dysfunctional (Giraldo et al. 2011; Zuckermann 1999; Parel & Chizzolini 2004). The propagation of US control PBMCs in the presence of plasma from Mali on occasion, did lead to slight, but likely functionally irrelevant, changes in the CD4+ and CD8+ frequencies, and PD-1 expression warranting further examination of plasma factors that may affect T cell homeostasis (Figure 4.7, 8).

To explore a potential therapeutic solution to the speculated immune dysfunction, we stimulated the PBMCs of one Mali donor following antibody-based blockade of PD-1 (Figure 4.9). Following anti-CD3 and anti-CD28 TCR triggering, we observed increases in the percentage of IFNγ-secreting CD4+ and CD8+ single-positive populations. We applied these preliminary findings to the assessment of PD-1 blockade during CSP-specific stimulation (Figure 4.10), and observed potential increased cytokine secretion.

These studies will be expanded by including additional donors, utilizing CSP-specific, MHC-I-restricted stimulation in HLA-A*02-positive donors.

We detected CSP-specific IFNγ responses from either stimulation with full-length CSP or HLA-A*02-restricted peptides, in four of 38 donors. This low frequency is comparable to other studies, and could be a result of both low frequency of specific cells, and the dysregulation of the T cell compartment we observed and speculated to be generally immune suppressive. PBMCs from two donors were HLA-A*02+ by surface staining and flow cytometry, and responded specifically to control and CSP-derived peptides. Therefore, the PBMCs from this donor could serve a useful tool for the studying of CSP-specific presentation to CTLs. They should be carefully examined for fine peptide-epitope specificities, and expanded if possible, for the generation of CSP-specific CTL lines.

Altogether, our findings on the frequencies of naturally induced antibody and T cell responses to CSP are in line with similar studies in other populations. It is important to conduct these immune-epidemiological studies in different populations from endemic areas, because each cohort is exposed to a different set of transmission dynamics, co-morbidities, and genetic diversity in parasites, and expresses varying HLA haplotypes. All these environmental and genetic factors are important in defining overall immune responses, as well as anti-malarial immunity. Understanding the development of effective anti-malarial immunity in endemic populations is crucial for designing a successful vaccine for this target population.

Chapter 5. CONCLUSIONS

5.1 Novel roles for traversed hepatocytes

5.1.1 Traversed hepatocytes as antigen depots

We have described novel findings relevant to CSP-specific T cell responses, which we propose to be meaningful for malaria vaccine development. In Chapter 2, we presented in-depth studies of sporozoite traversal-mediated deposition of leading vaccine candidate, CSP, into hepatocytes. Notably, these analyses were performed in an *in vitro* model using human hepatocytes and the most lethal parasite afflicting humans, *P. falciparum*. While this model is infrequently utilized in the laboratory due to its technical and biosafety-related challenges, we believe it is appropriate to studying both cell biology and immunology of human malaria infection.

We showed that hepatocytes traversed by sporozoites *in vitro* are viable, retain CSP for an extended duration with dynamic subcellular localization, and degrade CSP using multiple proteolytic machineries. We suggest that searches for other antigens potentially deposited into traversed hepatocytes should be conducted as well. The retention of sporozoite- and liver-stage antigen in traversed, but non-invaded cells offer the intriguing possibility of presentation to CTLs in the environment of the liver located close to invaded cells with developing parasites, the desirable target of cytotoxicity or parasite control.

5.1.2 Traversed hepatocytes as non-professional antigen presenting cells

The liver is characterized overall as immune tolerant. Perhaps, this is one of the reasons why *Plasmodium* sporozoites evolved to develop in the mammalian liver.

However, data do remain controversial on T cell activation and effector functions within this organ (reviewed in (Crispe 2009)). Having shown that traversed cells contain at least one malarial antigen, we evaluated the antigen presenting capacity of traversed hepatocytes as well as their potential to alter effector function of nearby CTLs, discussed in Chapter 3. We found that traversed HC-04 hepatocytes maintained unaltered levels of MHC class I and co-stimulatory ICAM and stimulated a full array of T cell activation programs upon antigen-specific, MHC class I-restricted stimulation. At least in this *in vitro* system, traversed cells exhibited no apparent defect or enhancement in the ability to stimulate CTL effector functions. Furthermore, utilizing a human CTL line specific for a C-terminal peptide of CSP, we showed that traversed cells could present this peptide epitope to induce IFNγ secretion. While this *in vitro* system of identification of traversed cells is technically unable to distinguish traversed cells that may contain parasites, possibly aborted or developing, we believe that this is the first demonstration of CSP presentation from traversed cells. The future use of rodent parasite models and parasites that are only able to invade but not traverse (Ishino et al. 2005) will allow further identification of the source of liver stage antigen to CTLs. The generation of additional CSP-specific CD8+ T cells would permit the determination of which other peptide epitopes may be presented from traversed, as well as infected hepatocytes.

We speculate on possible implications of the presentation of malarial antigens from traversed hepatocytes in the liver. *In vitro*, we observe far greater numbers of traversed than invaded hepatocytes. If this finding is recapitulated *in vivo* situation at least partially, then the presence of traversed cells presenting malarial antigens may increase overall activation of T cells specific for shared antigens present in both traversed

and invaded hepatocytes. This could, consequently, lead to greater secretion of cytokines IFNγ and TNFα, which are known to be important in limiting infection. Alternatively, traversed cells could divert and engage malaria-specific CTLs as decoys, limiting direct killing of infected hepatocytes. We expect that exciting developments in intravital imaging studies in the near future may help elucidate the architecture of traversed and invaded cells in the liver, and in turn, the dynamics of CTL recruitment and engagement with both populations of hepatocytes. If traversal serves an overall benefit to the host in controlling EEF development, such findings would support the further investment in generation of attenuated sporozoite vaccines that could traverse hepatocytes.

Importantly, our speculation on traversed hepatocytes as antigen presenting cells relates purely to the activation of memory T cells; we do not posit a role for traversed hepatocytes in priming of CD8+ T cells.

5.2 Consideration of existing immunity in vaccine development

We focused our studies on CTL responses against CSP because there is a tremendous amount of data from experimental models of malaria vaccination that demonstrate crucial roles for CD8+ immunity to liver stage, and CSP in particular. However, in developing a vaccine, it is essential to consider the immune status of the target population receiving the vaccine. The acquisition of partial but incomplete immunity to malaria in endemic regions has been noted throughout history. Children under the age of five are generally the victims of most severe disease and fatality. With continued exposure, however, the gradual development of partial immunity protects against severe disease. Considering the most clinically advanced vaccine, RTS,S, is only somewhat efficacious, interesting and concerning questions remain as to the

consequences of administering a partially protective vaccine in an endemic region: would it follow that it would partially reduce malaria burden, or have the opposite, potentially dangerous effect, of interfering with the development of naturally acquired immunity that is known to be protective, increasing the disease burden, doing more harm than good?

It is clear that immune responses to natural exposure and vaccination vary, in the degree of protection, as well as in the underlying immune mechanisms induced. Part of our study (Chapter 4), revealing a lack of correlation between anti-CSP IgG titers in naturally exposed donor plasma, and inhibition of sporozoite motility, illustrate this point. In vaccination studies, there is a dose-dependent relationship between inhibition of sporozoite motility by both monoclonal antibodies against CSP as well as the concentration of total IgG from vaccinated, protected individuals. This suggests that the neutralizing capacity of anti-sporozoite antibodies differ in natural exposure and in vaccination, in agreement with findings by Hoffman and colleagues (Hoffman et al. 1987). As we found evidence of dysregulated T cell responses in healthy adults residing in malaria endemic area, manifested as increased CD4+CD8+ populations in peripheral blood and PD-1 expression, as well as lower than expected responses to stimulation with viral peptides, we speculate that a vaccine should incorporate components to overcome the naturally regulatory responses to try to create a more protective, unnatural immune response.

Altogether, our studies advance the relatively recent understanding of sporozoite traversal through hepatocytes, suggesting potential roles for traversed hepatocytes in CD8+ T cell immunity. We also comprehensively investigated humoral responses and their functions in sporozoite infection, detected cellular responses specific to CSP, and

characterized phenotype and responsiveness of T cells circulating in peripheral blood of naturally exposed adults in Mali.

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CURRICULUM VITAE

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EDUCATION

2009-15 ***Johns Hopkins Bloomberg School of Public Health***, Baltimore, MD
Dissertation title: *Plasmodium falciparum* circumsporozoite protein: target of cellular immunity against traversed hepatocytes and naturally induced memory responses (research advisor: Dr. Jelena Levitskaya)

Ph.D. candidate in Department of Molecular Microbiology and Immunology

Relevant coursework:
Fundamentals of Virology; Principles of Immunology; Public Health Ecology; Pathogenesis of Bacterial Infections; Pandemics of the 20th Century; Biology of Parasitism; Food- and Water-borne Diseases; Emerging Infections; Host-Pathogen Interactions; Fundamentals of Epidemiology; Epidemiology of Emerging Infections; Clinical and Epidemiologic Aspects of Tropical Diseases; Field Studies in Ecology and Behavior (Valles Caldera National Preserve, New Mexico); Molecular Entomology; Principles of Human Nutrition; Infection, Immunity, and Undernutrition; Computational Biology and Bioinformatics; Molecular Biology and Genomics; Cell Structure and Dynamics; Fundamentals of Human Physiology; Animal Research: Law, Policy, and Human Sciences; Statistics for Laboratory Scientists; Statistics for Genomics; Research Ethics; Grant Writing

2004-09 ***University of Rochester,*** Rochester, NY
B.S. in Microbiology and Immunology
B.A. in Music, concentration in flute performance
Awarded a fifth-year tuition free for broadening studies as a Take Five Scholar in Jewish Culture
Cum laude

RESEARCH EXPERIENCE

Doctoral Research:

- Project description: *In vitro* analysis of infection of human hepatocytes with *Plasmodium* sporozoites to understand the role of sporozoite migration through hepatocytes in the anti-malarial CD8+ T cell response. Investigated breadth and

function of anti-CSP antibody and CD8+ T cell responses induced by natural infection in malaria-endemic Mali.

Doctoral Rotation Projects:

3/2010 – 5/2010, Ectopic expression of *Plasmodium falciparum* circumsporozoite protein in hepatocytes. (advisor: Dr. Jelena Levitskaya)

12/2009 – 3/2010, Innate immune response to *Plasmodium falciparum* in aseptic *Anopheles gambiae*. (advisor: Dr. George Dimopoulos)

9/2009 – 11/2009, Development of a live, oral Adenovirus vaccine against *Plasmodium falciparum* malaria. (advisor: Dr. Gary Ketner)

Undergraduate Research:

2007 – 09, Department of Microbiology and Immunology, University of Rochester School of Medicine and Dentistry, Rochester, NY (advisor: Dr. Stephen Dewhurst)

- Project Titles: Bacteriophage lambda as a vaccine platform against HIV-1
  Nanoparticle delivery of radioprotective siRNA in oral carcinomas

PUBLICATIONS

Ma J, **Trop S**, Baer S, Rakhmanaliev E, Arany Z, Dumoulin P, Zhang H, Romano J, Coppens I, Levitsky V, Levitskaya J. (2013) Dynamics of the major histocompatibility complex class I processing and presentation pathway in the course of malaria parasite development in human hepatocytes: implications for vaccine development. *PLoS One*. **9:** e75321.

Blumberg BJ, **Trop S**, Das S, Dimopoulos G. (2013) Bacteria- and IMD pathway-independent immune defenses against *Plasmodium falciparum* in *Anopheles gambiae*. *PLoS One.* **9:** e72130.

TECHNICAL SKILLS

- Molecular and cell biology: mammalian tissue culture – primary human hepatocytes, lymphocytes, T cell expansion, clinical samples, cell lines; immunofluorescence microscopy, western blot, antibody purification, PCR, DNA/RNA isolation, qRT-PCR, plasmid purification, transfection, microarray, virus titering
- Immunology: Flow cytometry, flow-assisted cell sorting, magnetic cell sorting, ImageStream imaging flow cytometry; T cell activation - ELISpot, cytokine secretion assay, intracellular cytokine staining, multiplex cytokine array; ELISA
- Parasitology: *Plasmodium* maintenance within and sporozoite isolation from *Anopheles* mosquitoes, mosquito dissection, handling of infectious BSL-3 materials
- Experimental design, data analysis, GraphPad Prism

SCIENTIFIC MEETINGS ORGANIZED

2013 - Chair, 2015 Gordon Research Seminar in Tropical Infectious Diseases, TX
Responsible for designing program, inviting speakers, obtaining funding through private sources, inviting and selecting participants for first time two-day Seminar for students and postdoctoral fellows preceding Gordon Research Conference

POSTER PRESENTATIONS

3/2015 Gordon Research Conference in Tropical Infectious Diseases, Galveston, TX
4/2013 World Malaria Day, Johns Hopkins Malaria Research Institute, Baltimore, MD
2/2013 Gordon Research Conference in Tropical Infectious Diseases, Galveston, TX
Awarded student scholarship
1/2013 Keystone Malaria Symposium, New Orleans, LA
4/2012 World Malaria Day, Johns Hopkins Malaria Research Institute, Baltimore, MD

SCIENTIFIC MEETINGS AND WORKSHOPS ATTENDED

11/2013 American Society for Tropical Medicine and Hygiene, Washington, DC
4/2012 New York Academy of the Sciences Malaria Conference, New York, NY
5/2010 American Society for Microbiology, San Diego, CA

SKILLS TRAINING

5/2014 Imagestream flow cytometry imaging software, 3 day training
6/2012 Confocal Microscopy Workshop, Clemson University
9/2009- Sommer Scholars Leadership program, Johns Hopkins
1/2006 Paychex Leadership Institute, Rochester, NY

TEACHING EXPERIENCE

2014 Teaching Assistant, Public Health Perspectives, Johns Hopkins
2011 Mentor for undergraduate summer research student, Johns Hopkins
2010 - 11 Teaching Assistant, Intro to Biomedical Sciences, Johns Hopkins
2005 - 07 Teaching Assistant, Intro to Biology, University of Rochester
2005 - 07 Biology Tutor, Office of Minority Student Assistance, University of Rochester

ACADEMIC SERVICE

2011 - Volunteer, University of Rochester Admissions
2009 - 12 Recruitment Committee, Admissions for Molecular Microbiology and Immunology Department, Johns Hopkins School of Public Health

HONORS AND AWARDS

2011 Ralph and Sylvia E. Barr Fellowship in Vector Biology

2010 Hegner, Cort, Root Memorial Fellowship in Immunology and Infectious Disease
2009 Sommer Scholar Leadership Award, Johns Hopkins
2009 Robert Woodbury Leadership Award, University of Rochester
2008 Take Five Scholar at the University of Rochester, Awarded a tuition-free year for broadening studies.
2008 Alice DeSimone Student Life Award, University of Rochester
2008 New York State College Health Association Student Recognition

PROFESSIONAL AFFILIATIONS
2013 - American Society for Tropical Medicine and Hygiene
2009 - American Society for Microbiology
2005 - 08 American College Health Association, Executive board member

LEADERSHIP EXPERIENCE
2009 - Co-founder and Secretary, Hopkins Honey
Established honeybee hive on Johns Hopkins Medical campus. Maintained hive, obtained funding, and organized educational events for Hopkins community about honeybees, food supply, and the environment.
2009 - Sommer Scholar Leadership Program, training in Public Health leadership
2005 - 07 President, Music Interest Floor, University of Rochester
2005 - 08 Chair, Student Health Advisory Committee, University of Rochester
2008 Student Co-chair, New York State College Health Association
2008 Upperclass Advisor, Sophomore Committee, University of Rochester